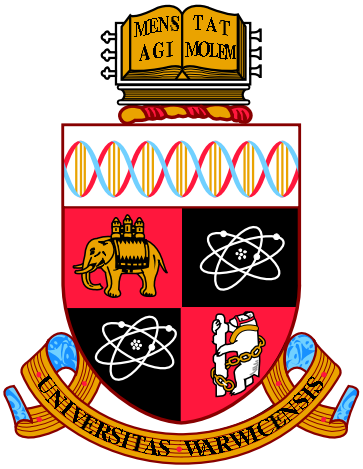

11β-HSD2 dependent and independent signalling pathways in trophoblast biology and glucocorticoid responsiveness

by

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Thesis

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Declarations

I hereby certify that this thesis (including data generated and data analysis) is my own work. All sources of information and contributions by others have been clearly disclosed and acknowledged. This thesis has not been submitted for a degree at another university.

Abstract

The placenta is the interface between the mother and the fetus fulfilling important roles such as nutrient transfer, hormone production and barrier functions. Pathological conditions associated with maternal stress during pregnancy can cause placental adaptations which possibly affect fetal health and might lead to long-term effects in later life. The placenta contains an autonomous endocrine system which consists of hormones such as corticotropin-releasing hormone (CRH) and cortisol (the human glucocorticoid) controlling stress responses. Exposure of the fetus and placenta to glucocorticoids (GCs) is finetuned by CRH. The presence of a GC barrier also modulates GC availability. The main component of this GC barrier is the enzyme 11β-HSD2 which catalyzes active cortisol into cortisone. Expression of this enzyme is decreased in placental diseases such as pre-eclampsia and intrauterine growth restriction (IUGR). This might be associated with elevated levels of trophoblast apoptosis and disturbances of trophoblast differentiation found in pregnancy-related disorders. Very little is known about the specific role of this enzyme and related pathways in placental apoptosis, differentiation, endocrine capacity and the expression of molecules involved in the stress response and glucocorticoid action. These potential effects were investigated in this thesis by using the placental explant culture and the choriocarcinoma BeWo cellular model. Furthermore, the capacity of GCs to regulate transcriptional events in BeWo cells was explored.
My results show that CRH treatment and activation of TLR4 have modulating effects on placental hCG production in placental explants. Moreover, the enzyme 11β-HSD2 seems to be involved in the homeostasis of placental differentiation and apoptotic processes, the maintenance of hCG and progesterone secretion and it might limit overactivation of CRH receptors and CRH secretion. GC insensitivity was identified in the BeWo cells and it appears that in these cells the cAMP pathway is the predominant pathway in regulating GC-responsive genes. Through a series of reporter-gene-assays my results suggest that transfection of exogenous GRα restores the BeWo cell sensitivity to GCs without affecting BeWo cell turnover and hormone secretion.
In conclusion, placental CRH and 11β-HSD2 expression appear to play an important role in the maintenance of placental endocrine function. GCs on the other hand did not have strong regulatory effects in the BeWo cells suggesting a unique mechanism through which trophoblast cells become insensitive to local glucocorticoid action.

Abbreviations

ABC	ATP-binding cassette
AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
ADAM	a disintegrin and a metalloproteinase domain
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
Apaf-1	apoptotic protease activating factor-1
APS	ammonium persulfate
ASCT2	ASC amino acid transporter 2
ATB0	amino acid transporter B 0
ATP	adenosine triphosphate
ATP1A1	ATPase subunit α 1
AVP	arginine vasopressin
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
BCRP	breast cancer resistance protein
BeWo-GRα	BeWo cells overexpressing GRα
BH	Bcl-2 homology domain
BLAST	basic local alignment search tool
BM	basal membrane
BSA	bovine serum albumin
°C	degree Celsius

cAMP	cyclic AMP
CD	cluster of differentiation
cDNA	complementary DNA
CMRL	Connaught Medical Research Laboratories
CO_2	carbon dioxide
CRE	cAMP response element
CREB	cAMP response element binding
CRH	corticotropin-releasing hormone
CRH-BP	CRH binding protein
CRH-R	CRH receptor
CSF	colony-stimulating factor
CT	cytotrophoblast
C_T	threshold cycle
CXC	cystein x cystein
CXCL	CXC ligand
CYP	cytochrome P450
DAB	diaminobenzidine
DAPI	4',6-Diamidino-2'-phenylindole
DC	dendritic cell
DBD	DNA binding domain
Dex	dexamethasone
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DISC	death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
Dusp1	dual specificity protein phosphatase 1
E2	estradiol

E-Cad	E-Cadherin
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
EVT	extravillous trophoblast
F	forward
FABP	fatty acid binding protein
FATP	fatty acid transport protein
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGR	fetal growth restriction
FSH	follicle-stimulating hormone
fsk	forskolin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid
GDM	gestational diabetes
GDP	guanosine diphosphate
GFP	green fluorescent protein
Gilz	glucocorticoid-induced leucine zipper
GLUT	glucose transporter
GM-CSF	granulocyte-macrophage CSF
GPCR	G protein coupled receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GTP	guanosine triphosphate
h	hour
h	human
HbA1c	hemoglobin A1c

hCG	human chorionic gonadotropin
HEK	human embryonic kidney
HeLa	Henrietta Lacks
	(immortal cell line derived from cervical cancer)
HELLP	haemolysis, elevated liver enzymes, low platelet
HERV	human endogenous retroviruses
HFA	human fetal adrenal
hGR	human GR
hMR	human MR
H_2O	water
HPA	hypothalamic pituitary adrenal
hPL	human placental lactogen
HRP	horse radish peroxidase
HSD	hydroxysteroid dehydrogenase
hsp	heat shock protein
ICM	inner cell mass
IGF	insulin-like growth factor
IHC	immunohistochemistry
IL	interleukin
IUGR	intrauterine growth restriction
LH	luteinizing hormone
LPS	lipopolysaccharide
luc	luciferase
mA	milliampere
MAPK	mitogen-activated protein kinase
MDR	multidrug resistance
MFSD2	major facilitator superfamily domain containing 2
μg	microgramm
min	minute
μL	microlitre

μM	micromolar
M	molar
mL	millilitre
mM	millimolar
MMP	matrix metalloproteinase
MMTV	mouse mammary tumor virus
MPTP	mitochondrial permeability transition pore
MR	mineralocorticoid receptor
MRP	multidrug resistance-associated protein
MVM	microvillous membrane
NAD+	nicotinamide adenine dinucleotide
NCoR	nuclear co-repressor
NFκB	nuclear factor κ B
NK	natural killer cell
NOS	nitric oxide synthase
NR3C1	nuclear receptor subfamily 3, group C, member 1
NR3C2	nuclear receptor subfamily 3, group C, member 2
ns	non significant
NTD	N-terminal domain
O_2	oxygen
OD	optical density
p	phosphorylated
P4	progesterone
p38	protein 38
p53	protein 53
PARP	poly-(ADP-ribose)-polymerase
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween 20
p.c.	post conception
PCR	polymerase chain reaction

Per1	period circadian protein homolog 1
PGD	pregestational diabetes
P-gp	P-glycoprotein
PIC	protease inhibitor cocktail
PI3K	phosphatidylinositide 3 kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PlGF	placental growth factor
PMSF	phenylmethylsulfonyl fluoride
Pol-II	polymerase II
pp53	phosphorylated p53
PR	progesterone receptor
PS	phosphatidylserine
PUMA	p53 upregulated modulator of apoptosis
qRT-PCR	quantitative reverse transcription-PCR
R	reverse
RDR	RD114/mammalian type D retrovirus receptor
RLU	relative light unit
Rluc	renilla luciferase
RNA	ribonucleic acid
ROS	reactive oxygen species
18S rRNA	18 Svedberg ribosomal RNA
RSV	Rous sarcoma virus
RT	reverse transcriptase
s	second
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
sFlt-1	soluble fms-like tyrosine kinase-1
SGK1	serum- and glucocorticoid-regulated kinase 1

siHSD2	11β-HSD2-siRNA
siRNA	small interfering RNA
ST	syncytiotrophoblast
STS	staurosporine
sVEGFR-1	soluble VEGF Receptor-1
TBS	tris buffered saline
TBST	TBS with Tween 20
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TGF	transforming growth factor
TLR	toll-like receptor
T_m	melting temperature
TMD	transmembrane domain
TNF	tumor necrosis factor
TNFR	TNF receptor
Treg	regulatory T cell
TSH	thyroid-stimulating hormone
TWB	tris wash buffer
U	unit
Ucn	urocortin
uNK	uterine NK
UTR	untranslated region
V	Volt
VEGF	vascular endothelial growth factor

Chapter 1

Introduction

1.1 Stress Response: CRH and Glucocorticoid Metabolism and 11β-HSD2

Stress during pregnancy is now considered to play a major role in fetal health and pregnancy outcomes. To investigate the effects of stress on tissues including the placenta, the hormonal mediators of stress responses corticotropin-releasing hormone (CRH) and cortisol (the human glucocorticoid) are of fundamental importance. This chapter focuses on the physiological roles of these two hormones and also on the enzyme 11β-HSD2 which is a modulator of the cellular glucocorticoid availability and which is highly expressed in the placenta.

1.1.1 HPA Axis

To maintain a homeostatic state and to be able to respond appropriately to physical, emotional and immunological stressors, organisms have developed special endocrine systems. The hypothalamic-pituitary-adrenal (HPA) axis regulates these responses (Figure 1.1). The key regulator molecules of the HPA axis are corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) which are synthesized by neurons located in the paraventricular nuclei of the hypothalamus [Swanson et al., 1983]. After reaching the anterior pituitary gland, CRH and AVP bind synergistically to their receptors in the corticotroph cells which stimulates synthesis and secretion of adrenocorticotropic hormone (ACTH) into the bloodstream [Orth et al., 1983; Watanabe and Orth, 1987; Pecori Giraldi and Cavagnini, 1998]. In the zona fasciculata of the adrenal gland, ACTH triggers the synthesis and systemic secretion of glucocorticoids which exert pleiotropic effects in many target tissues due to the ubiq-

uitous expression of the glucocorticoid receptor (GR) [Kemppainen and Behrend, 1997]. The glucocorticoids act in a negative feedback loop on the level of the hippocampus, hypothalamus and the pituitary inhibiting CRH and ACTH production [Jacobson and Sapolsky, 1991; Mahmoud and Jones, 1977; Jones et al., 1977]. This mechanism ensures a tight control and prevents a prolonged hyperactivation of the HPA-axis.

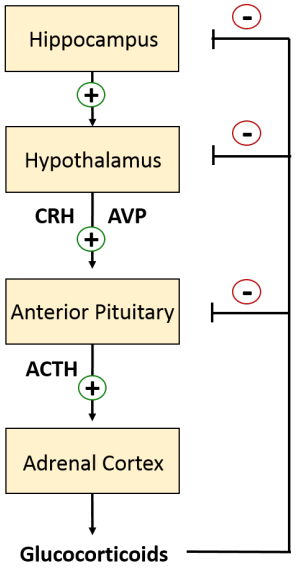

Figure 1.1: **Diagram of the HPA axis.** Stressful stimuli trigger expression of CRH and AVP in the hypothalamus. These two hormones provoke synthesis of ACTH in the pituitary gland. ACTH then leads to glucocorticoid secretion in the adrenal cortex. Glucocortiocoids exert various effects in target tissue and a negative feedback effect on the hippocampus, hypothalamus and pituitary. Adapted from Boonstra [2004].

1.1.2 Corticotropin-Releasing Hormone (CRH)

In 1981, the 41 amino acid peptide CRH was isolated by Vale et al. [1981]. CRH belongs to a family of related peptides, which also includes the mammalian peptides Urocortin I (Ucn I), Ucn II and Ucn III, fish urotensin and frog sauvagine [Vaughan et al., 1995; Reyes et al., 2001; Lewis et al., 2001; Lederis et al., 1982; Montecucchi et al., 1980]. CRH is the main regulator of the stress response. In addition to the hypothalamus, CRH is also produced by various tissues including the placenta. At inflammatory sites, CRH seems to be an important regulator [Scopa et al., 1994; Mastorakos et al., 1995] and also in the reproductive tissues, CRH is involved in inflammatory-like phenomena such as blastocyst implantation and early immune tolerance [Makrigiannakis et al., 2001, 2004]. In case of hypersecretion, CRH seems to be implicated in various diseases such as anxiety disorders, depression and metabolic syndrome [Holsboer, 1999]. A circulating binding protein, called CRH-binding protein (CRH-BP), is produced by the liver and placenta and it can inactivate CRH [Linton et al., 1988; Potter et al., 1991; Sehringer et al., 2004]. This subchapter introduces the CRH modes of action and the regulatory effects of CRH with a special focus on reproductive roles.

1.1.2.1 CRH Receptors and Signalling

CRH can bind to two CRH receptors, namely CRH-R1 and CRH-R2. These receptors belong to the family of seven transmembrane domain (7TMD) G-protein coupled receptors (GPCR) [Chang et al., 1993] and they are encoded by two different genes located on chromosome 17 and 7, respectively [Vamvakopoulos and Sioutopoulou, 1994; Meyer et al., 1997]. The homology between CRH-R1 and CRH-R2 is 71% on amino acid level [Lovenberg et al., 1995]. Because of alternative splicing, several receptor subtypes for both CRH receptors are expressed in various tissues, namely CRH-R1α, -R1β, -R1c, -R1d, -R1e, -R1f, -R1g, -R1h, and CRH-R2α, -R2β, -R2γ [Chen et al., 1993; Ross et al., 1994; Grammatopoulos et al., 1999; Pisarchik and Slominski, 2001; Liaw et al., 1996; Valdenaire et al., 1997; Kostich et al., 1998]. The N-terminus of the CRH receptors is involved in ligand binding and selectivity [Perrin et al., 1998; Wille et al., 1999]. The peptides CRH, Ucn I, urotension I and sauvagine bind to the CRH-R1 with equal affinity and the peptides Ucn I, Ucn II, Ucn III, urotensin I and sauvagine bind to the CRH-R2 with a higher affinity than CRH binds to this receptor [Grammatopoulos and Chrousos, 2002].

The CRH receptors preferentially couple to the G_s-adenylyl cyclase signalling

pathway, but also exhibit alternative coupling to and activation of other G-proteins such as G_i, G_o, G_q, and G_z exists (Figure 1.2) [Chen et al., 1986; Grammatopoulos et al., 2001]. Further downstream signalling molecules include the protein kinases A (PKA), PKC, MAP kinases p38 and $ERK_{1/2}$ and also signalling molecules such as Ca^{2+} and nitric oxide synthase (NOS) [Ulisse et al., 1990; Brar et al., 2004; Kiang, 1997; Cantarella et al., 2001]. The variety of CRH receptor splice variants and possible signalling pathways allow CRH to evoke different responses in a tissue-dependent manner. Interestingly, in the placenta no coupling of the CRH receptors to the G_s protein is observed, even though G_s protein is expressed [Karteris et al., 2000]. Instead, coupling of CRH receptors to G_q, G_o, and G_z takes place in the placenta [Karteris et al., 2000].

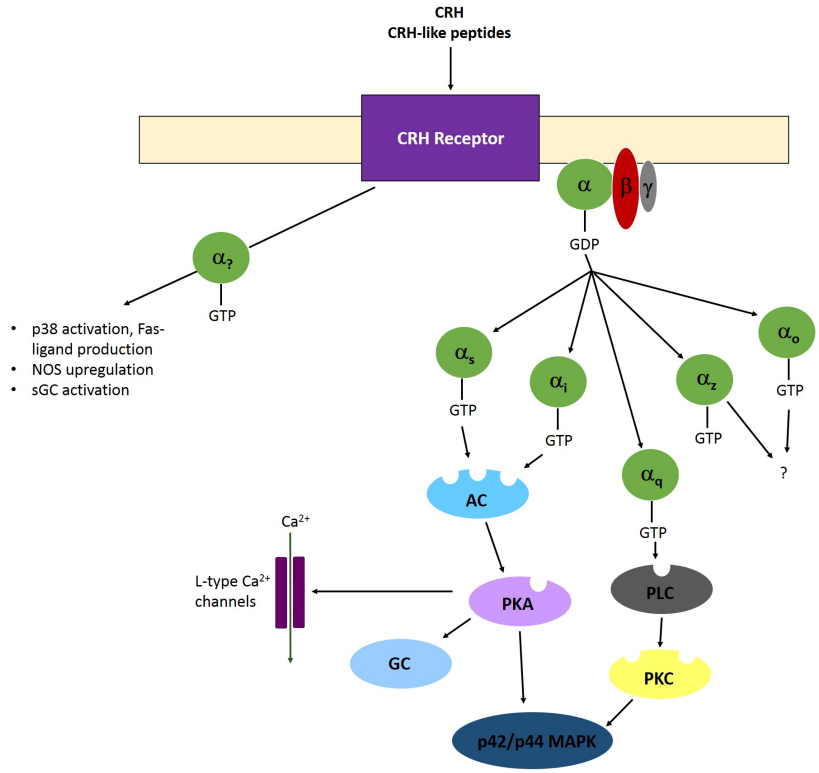

Figure 1.2: **Signalling of the CRH receptor.** A variety of signalling pathways can be activated upon CRH binding to its receptor in a tissue-dependent manner. AC = adenylyl cyclase, GC = guanylyl cyclase, GDP = guanosine diphosphate, GTP = guanosine triphosphate, MAPK = MAP kinase, NOS = nitric oxide synthase, PKA = protein kinase A, PKC = protein kinase C, PLC = phospholipase C, sGC = soluble GC. Adapted from Grammatopoulos and Chrousos [2002].

1.1.2.2 CRH in Reproductive Tissues

Grino et al. [1987] showed that CRH is expressed by the human placenta and Jones et al. [1989] further demonstrated that CRH is also expressed in amnion, chorion and decidua. The placental localization of CRH was investigated by Riley et al. [1991] who showed a CRH-immunoreactivity in the syncytiotrophoblast, but not in cytotrophoblasts.

The placental CRH, which is identical with the hypothalamic CRH, is located on chromosome 8 [Arbiser et al., 1988]. Interestingly, CRH expression exponentially rises during pregnancy only in humans and higher primates such as rhesus monkeys and gorillas [Robinson et al., 1989] suggesting a distinct and specific regulation of pregnancy of these species by CRH. In contrast to hypothalamic CRH, the placental CRH production is not suppressed by glucocorticoids, in fact glucocorticoids increase expression and secretion of placental CRH [Robinson et al., 1988]. That suggests presence of a positive feedback loop between CRH and glucocorticoids in the placenta. In addition to the glucocorticoid regulation of CRH secretion, cAMP stimulators have been shown to increase CRH expression in primary trophoblast cells [Cheng et al., 2000].

The concentration of CRH rises towards the end of pregnancy [Frim et al., 1988]. Furthermore, the concentration of CRH-BP falls within the third trimester of pregnancy which leads to higher concentrations of active CRH towards the end of pregnancy [Linton et al., 1993]. In pregnancy-related disorders such as pre-eclampsia, intrauterine growth restriction (IUGR), and pre-term labour, CRH levels rise higher than during normal pregnancies [Laatikainen et al., 1991; Goland et al., 1993; Warren et al., 1992].

Placental CRH appears to play several roles during pregnancy. By activating nitric oxide synthase CRH leads to vasodilatation of uterine and placental vessels which is important for maintaining the vascular tone in the feto-placental circulation [Clifton et al., 1995]. Placental CRH plays a role in parturition as it stimulates the secretion of prostaglandin $F_{2\alpha}$ and E_2 and modulates prostaglandin $F_{2\alpha}$ activity which leads to contraction of myometrial cells [Jones and Challis, 1989; Benedetto et al., 1994]. Furthermore, placental CRH regulates parturition by increasing matrix metalloproteinase-9 (MMP-9) activity which contributes to membrane rupture [Li and Challis, 2005].

In the fetal adrenal zone of the adrenal gland, placental CRH leads to the production of dehydroepiandrosterone and its sulfate (DHEA/DHEAS) [Smith et al., 1998a]. The human fetal adrenal (HFA) and the placenta form the so-called feto-placental unit. The DHEA/DHEAS produced by the HFA are supplied to the

placenta as precursor molecules to promote the production of estrogens [Siiteri and MacDonald, 1966; Sirianni et al., 2005a]. Moreover, CRH also induces cortisol production in the fetal adrenal cells which then contributes to maturation of fetal organs [Sirianni et al., 2005b].

McLean et al. [1995] suggested the concept that CRH acts as a placental clock. They discovered that CRH concentration were higher in women delivering pre-term and lower in women delivering post-term compared to normal pregnancies.

CRH is further expressed in endometrial cells and ovaries. In the endometrium, epithelial cells and decidualized stromal cells express CRH which plays an important role in the implantation process [Makrigiannakis et al., 1995; Mastorakos et al., 1996]. Ferrari et al. [1995] showed that CRH leads to the decidualization of stromal cells which is a prerequisite for a successful implantation of the blastocyste. The effect of progesterone, main inducer of decidualization, is modulated by interleukin-1 and -6 (IL-1, IL-6) which in turn are shown to be influenced by CRH [Zoumakis et al., 2000]. In this way, CRH may fine-tune the decidualization process.

During the early stages of the implantation of the blastocyst, a maternal immunological tolerance is established. CRH plays a role in this process by increasing apoptosis of activated T lymphocytes [Makrigiannakis et al., 2001].

During the invasion of the extravillous trophoblast cells, CRH controls this invasion by downregulating the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) expression [Bamberger et al., 2006].

In the ovaries, CRH is expressed in the thecal cells surounding the ovarian follicles and in luteinized cells of the stroma [Mastorakos et al., 1994]. More CRH is detected in thecal cells of mature follicles than in small follicles which might implicate a role of CRH in the follicular maturation [Asakura et al., 1997]. Regarding steroidogenesis, CRH inhibits estradiol and progesterone production by human granulosa cells [Ghizzoni et al., 1997].

1.1.3 Regulators of Glucocorticoid Action

1.1.3.1 Glucocorticoid Receptor

The ubiquitously expressed glucocorticoid receptor (GR) belongs to the steroid/thyroid/retinoic acid nuclear receptor family of ligand-dependent transcription factors. The human (h) GR gene NR3C1 (nuclear receptor subfamily 3, group C, member 1) is located on chromosome 5, has nine exons and several mRNA isoforms are transcribed because of alternative splicing [Encío and Detera-Wadleigh, 1991]. The

hGR was first cloned by Hollenberg et al. [1985] identifying two homologous protein isoforms, GRα and GRβ, which only differ in their exon 9 (Figure 1.3). In addition, multiple other isoforms of GRα exist due to alternative translation initiation sites [Lu and Cidlowski, 2005]. The protein structure of the two GR isoforms GRα and GRβ consists of an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) [Giguère et al., 1986] (Figure 1.3). The transactivation domain AF-1 is located in the NTD functioning in a hormone-independent way, the DBD binds to DNA via zinc-finger motifs and is involved in the dimerization of two GRα molecules, and the LBD binds the ligands and contains a second transactivation domain, AF-2, which is hormone-dependent [Luisi et al., 1991; Hollenberg and Evans, 1988]. The GRα form is the isoform which acts as the ligand-activated transcription factor, whereas the GRβ isoform does not bind agonists and can inhibit the transcriptional action of GRα [Bamberger et al., 1995].

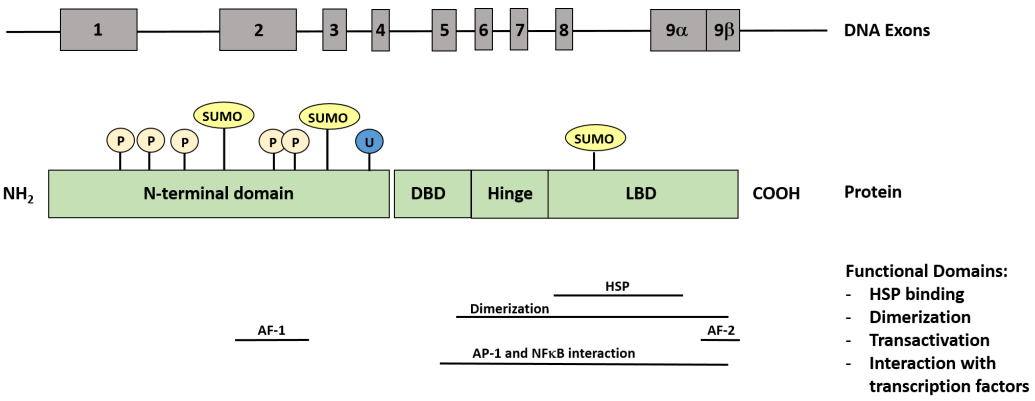

Figure 1.3: **Diagram of GR domains.** The GRα and GRβ isoforms are produced by alternative splicing of exon 9. The protein structure is divided into the NTD, DBD, hinge region and LBD which are involved in different functions such as binding to heat shock proteins, transactivation of the GR, receptor dimerization, DNA binding at glucocorticoid-response elements, and interaction with other transcription factors. Post-translational modification sites of the GR are labelled with P, SUMO and U. DBD = DNA-binding domain, HSP = heat shock protein, LBD = ligand-binding domain, NTD = N-terminal domain, P = phosphorylation, SUMO = sumoylation, U = ubitquitination. Adapted from McMaster and Ray [2008].

In the absence of ligand, the GRα is localized in the cytoplasm and forms a hetero-oligomeric complex with several other proteins such as heat-shock proteins (hsp) and immunophilins [Dalman et al., 1991; Tai et al., 1992; Pratt and Toft, 1997]. Hsp90 of this complex is of particular interest as it masks the nuclear localization sequences (NLS) of the GRα and keeps the GRα in an inactive conformation that allows ligand binding [Bresnick et al., 1989; Cadepond et al., 1991]. After binding of ligand to the LBD, the conformation of the GRα changes, it dissociates from the Hsp90 and the hetero-oligomeric complex and translocates to the nucleus [Picard and Yamamoto, 1987]. Two GRα molecules homodimerize and bind to glucocorticoid-responsive elements (GREs) in promoter regions of target genes and thereby lead to activation or repression of these genes [Tsai et al., 1988; Wrange et al., 1989].

During transcriptional activity, GRα interacts, via its AF-1 and AF-2 domains, with several coactivators (Figure 1.4) such as p300/CBP (=p300 and cAMP-responsive element-binding protein (CREB)-binding protein), the p/CAF (=p300/-CBP associated factor), the p160 family of coactivators which include the SRCs (=steroid receptor coactivators), the SWI/SNF (=SWItch/sucrose non-fermenting) complex, and the complex DRIP/TRAP (=vitamin-D-receptor interacting protein/-thyroid receptor-associated protein) [Chakravarti et al., 1996; Oñate et al., 1995; Li et al., 2003; Hittelman et al., 1999]. The coactivators have chromatin remodeling activity or facilitate the binding of the basal transcription machinery.

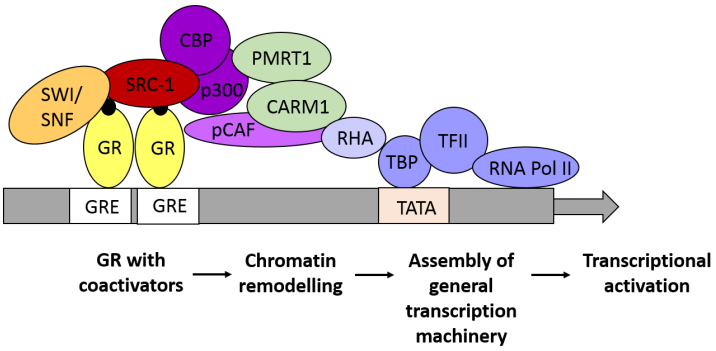

Figure 1.4: **Diagram of GR with its coactivators.** GR interacts with several coactivators such as SRC1, p300, CBP, SWI/SNF and p/CAF. PRMT1 and CARM1 = histone methyltransferases, RHA = RNA helicase, TBP = TATA-box binding protein. TFII = general transcription factor II, RNA Pol II = RNA polymerase II. Adapted from Walsh et al. [2012].

Also posttranslational modifications of the GRα such as phosphorylation, acetylation, sumoylation and ubiquitination have an influence on the GR transcriptional activity [Ismaili and Garabedian, 2004; Nader et al., 2009; Le Drean et al., 2002; Kinyamu et al., 2005].

In addition to acting as a homodimer on GRE elements of target genes to induce gene transcription, the GRα can also interact with other transcription factors such NFκB, AP-1 and STATs and thereby regulating gene transcription via transcriptional cross-talk (Figure 1.5 for the various possibilities of GR mechanisms) [Scheinman et al., 1995; Karin and Chang, 2001; Rogatsky and Ivashkiv, 2006].

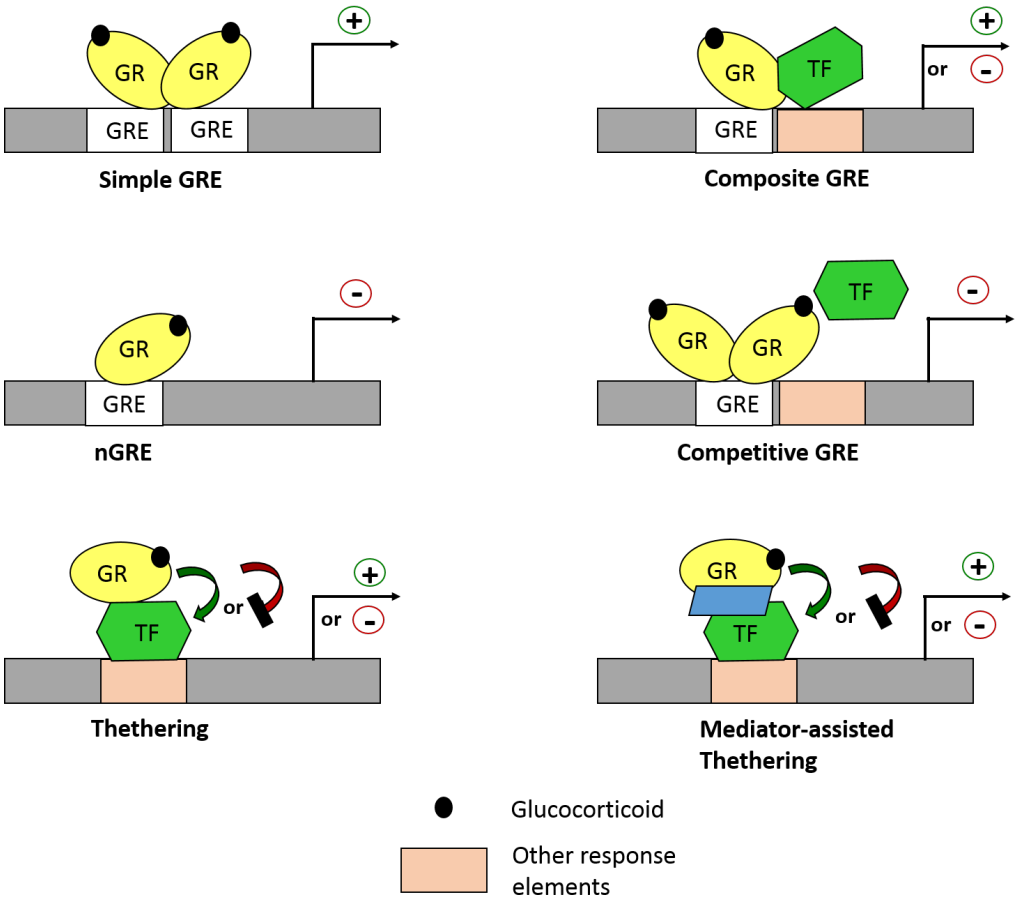

Figure 1.5: **Diagram of GR genomic mechanisms.** The ligand-bound GR activates or represses transcription of target genes via several genomic mechanisms. GR = glucocorticoid receptor, GRE = glucocorticoid response element, TF = transcription factor. Adapted from Ratman et al. [2013].

Non-genomic actions of GCs

Glucocorticoids can also exert effects via three different non-genomic mechanisms. Glucocorticoids can intercalate into membranes which leads to changes in their physicochemical properties and influences cation transport [Stahn and Buttgereit, 2008]. Rapid effects of glucocorticoids, which do not involve protein synthesis, might be explained by this model. Furthermore, glucocorticoids can bind to membrane-bound glucocorticoid receptors (= mGRs). There are two types of mGRs: the classic GR localized in the membrane and a non-classic mGR. These mGRs evoke responses via MAPK and cAMP signalling pathways [Mitre-Aguilar et al., 2015]. Also, after binding of glucocorticoids to the cytosolic classic GR, proteins such as heat shock proteins and cochaperones are released which can exert non-genomic signalling effects [Japiassú et al., 2009].

1.1.3.2 Mineralocorticoid Receptor

The MR belongs to the same steroid receptor superfamily as the GR and acts as a ligand-activated transcription factor as well. The MR is encoded by the gene NR3C2 and was cloned by Arriza et al. [1987]. The endogenous ligand of the MR is aldosterone, but the MR has similar affinity to cortisol [Arriza et al., 1987]. Because of this fact, effects of glucocorticoids can also be mediated by MR in addition to GR. Like the GR, the MR has an N-terminal domain, a DNA-binding (DBD), a hinge region and a ligand-binding domain. The DBD from the MR is 94% homolog to the DBD from the GR and binds to GRE elements in target gene promotors [Lombés et al., 1993].

The MR is expressed in mineralocorticoid-target tissue including kidney and in other tissues such as colon, salivary glands, specific brain regions, vascular tissue, adipocytes and placenta [Lombès et al., 1990; Sasano et al., 1992; Agarwal et al., 1993; Hirasawa et al., 2000; Pascual-Le Tallec and Lombès, 2005]. In mineralocorticoid-target tissue, the enzyme 11β-HSD2 is very important because it inactivates cortisol to cortisone and thereby enhances the binding of aldosterone to the MR (see chapter 1.1.5).

Similar to GR trafficking, the MR forms a heterocomplex with other proteins such as Hsp90, Hsp70, p23 and immunophilins in the cytosol [Nemoto et al., 1993; Gallo et al., 2007], but also some nuclear localization of the MR is detectable [Nishi et al., 2001]. After ligand binding, the MR translocates to the nucleus, possibly with Hsp90, and within the nucleus MR dimerizes and transcription of target genes

can occur [Galigniana et al., 2010; Grossmann et al., 2012].

1.1.4 Placental and Fetal GC Action

Fetal cortisol partly stems from the mother's circulation by reaching the fetus via transplacental transfer and is also partly produced by the fetus itself, specifically it is produced by the fetal zone (FZ) from the fetal adrenal gland. Fetal cortisol levels increase from week 35 of pregnancy until term [Murphy, 1982] which leads to fetal organ maturation preparing the fetus for extrauterine life.

Cortisol plays an important role on the placental development as it can regulate placental size and morphology as well as it is involved in the regulation of placental transport and endocrine function [Fowden et al., 2009]. Furthermore, cortisol might act as sensor important for initiating adaptive responses in the placenta [Fowden and Forhead, 2004].

Also, cortisol plays an important role in fetal organ maturation to prepare the fetus for extrauterine life. For example, cortisol is involved in lung maturation, in detail it leads to stimulation of surfactant protein production which is required for a successful extrauterine function of the lung [Liley et al., 1989]. A single dose of synthetic glucocorticoid is the standard therapy for pregnant women with risk of pre-term labor as it accelerates the fetal lung maturation and thereby reduces the morbidity and mortality caused by infant respiratory distress syndrome [Roberts and Dalziel, 2006]. Furthermore, cortisol is important for the induction of hepatic gluconeogenesis enzymes, for cardiac development, and for establishing an independent thermoregulation [Townsend et al., 1991; Fowden et al., 1998; Reynolds and Walker, 2003]. Thus, cortisol is very important for the successful maturation of the fetus, but an excess of cortisol on the other hand can have detrimental effects on fetal development such as intrauterine growth restriction and can lead to fetal programming (see chapter 1.3.3).

Figure 1.6 summarizes the CRH and glucocorticoid signalling in the mother, placenta and fetus.

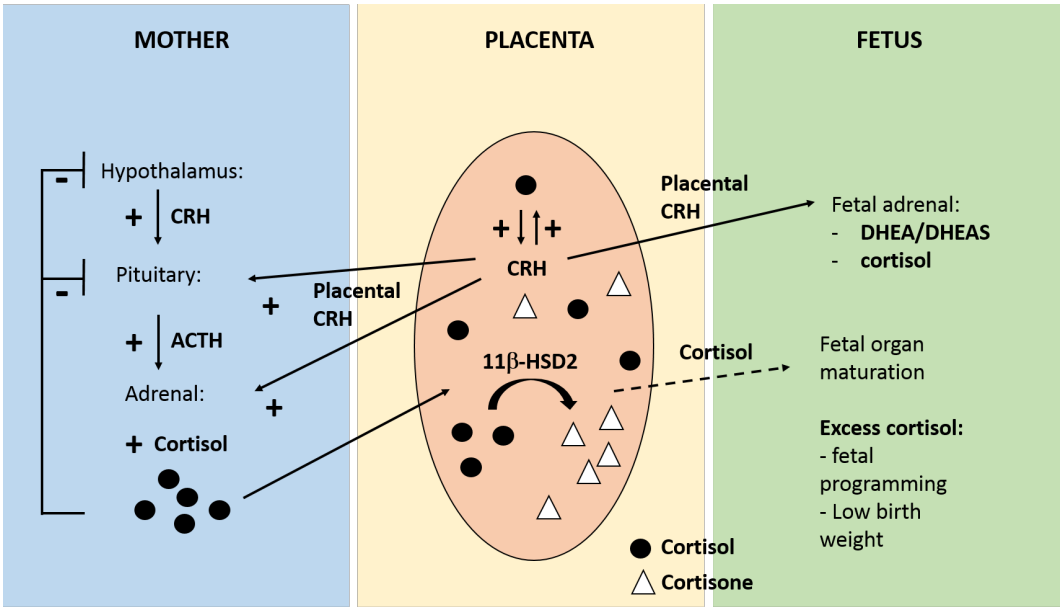

Figure 1.6: **Diagram of CRH and GC signalling between mother, placenta and fetus.** Maternal cortisol (human glucocorticoid) levels are increased after activation of the maternal HPA axis. Cortisol molecules pass through the placenta where they are being inactivated by the enzyme 11β-HSD2 to cortisone (see chapter 1.1.5 for detailed information about 11β-HSD2). However, also local glucocorticoid action in placental tissue takes place, e.g. cortisol increases placental CRH levels and cortisol regulates placental development. Placental CRH activates production of fetal DHEA/DHEAS (precursor of E2 in the placenta) and fetal cortisol in the fetal adrenal. Fetal and maternal cortisol are important for fetal organ maturation, but excess cortisol levels in the fetus can lead to fetal programming and low birth weight (see chapter 1.3.3 for detailed information about fetal programming). Adapted from Reynolds [2013a].

1.1.5 The Enzyme 11β-HSD2

The enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) is a modulator of glucocorticoid availability in cells playing an important role in mineralocorticoid-target tissues as well as in the placenta.

The isoform 11β-HSD2 was found and characterized by Brown et al. [1993] and Rusvai and Náray-Fejes-Tóth [1993] and cloned by Albiston et al. [1994]. 11β-HSD2 catalyzes exclusively the conversion of cortisol and corticosterone to cortisone and 11-dehydrocorticosterone, respectively (Figure 1.7). Thereby NAD^+ is required as a cofactor in this biochemical reaction. In contrast to that, the isoform 11β-HSD1 catalyzes mainly the conversion of cortisone to cortisol which requires NADPH as a cofactor [Low et al., 1994]. However, this type I enzyme also has a dehydrogenase activity, even though the reductase activity is predominant. Sequence homology (amino acids) between 11β-HSD1 and 11β-HSD2 is only 14% [Albiston et al., 1994].

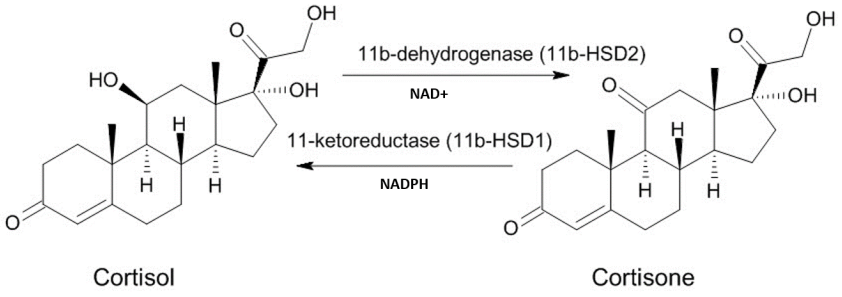

Figure 1.7: **Biochemical reactions of 11β-HSD1 and 11β-HSD2.** 11β-HSD2 catalyzes the inactivation of cortisol into cortisone and 11β-HSD2 catalyzes the conversion of cortisone to cortisol. Adapted from http://www.totalflexblog.com/articles/11b-hsd1-inhibition/ .

The enzyme 11β-HSD2 is expressed in classical aldosterone/mineralocorticoid-target tissues including kidney and colon as well as in the placenta [Krozowski et al., 1995; Smith et al., 1996]. As mentioned above (see chapter 1.1.3.2) the MR binds cortisol and aldosterone with equal affinity *in vitro*, but *in vivo* the MR selectively binds aldosterone. This is because of the activity of 11β-HSD2 inactivating cortisol and its colocalization with the MR [Edwards et al., 1988; Funder et al., 1988; Hirasawa et al., 1997; Odermatt et al., 2001].

11β-HSD1 is expressed highest in liver [Tannin et al., 1991] where it is colocalized with the GR modulating cortisol access to this receptor [Low et al., 1994].

Role of 11β-HSD2 in Placenta

Expression of a dehydrogenase (11β-ol dehydrogenase) in the placenta which converts cortisol into cortisone was described by Osinski [1960]. The now called 11β-HSD2 plays an important role as the placental glucocorticoid barrier enzyme, which limits the maternal cortisol to reach the fetus by inactivating the active cortisol into cortisone [Benediktsson et al., 1997]. This mechanism is extremely efficient as it has been shown that the cortisol concentration in the maternal circulation is 5- to 10-fold higher than in the fetal compartment [Beitins et al., 1973; Campbell and Murphy, 1977]. When excess glucocorticoids reach the fetus, for example due to stress or a decreased 11β-HSD2 expression, it could lead to a reduced birth weight and fetal programming (see chapter 1.3.3), i.e. the individuals have a higher risk in developing diseases such as metabolic and anxiety-related diseases in their adult life [Seckl and Holmes, 2007].

The enzyme 11β-HSD2 is highly expressed in the syncytiotrophoblast layer, which is the maternal-fetal interface, and weakly expressed in the cytotrophoblast cells [Krozowski et al., 1995; Driver et al., 2001]. The placental 11β-HSD2 expression steadily increases towards the end of pregnancy, whereas during the last two weeks of gestation the 11β-HSD2 activity decreases [Schoof et al., 2001b; Murphy and Clifton, 2003]. In placenta-related diseases such as pre-eclampsia and IUGR, expression of 11β-HSD2 is reduced (see chapter 1.3.1) [Schoof et al., 2001a; Causevic and Mohaupt, 2007; Shams et al., 1998; Dy et al., 2008; McTernan et al., 2001].

Regarding transcriptional regulation, upregulation of 11β-HSD2 during syncytialization of the cytotrophoblast cells is due to an elevated expression of the transcription factor Sp1 [Li et al., 2011a]. The MAP kinases p38 and $ERK_{1/2}$ are involved in the up- and downregulation of basal 11β-HSD2 expression, respectively [Sharma et al., 2009; Guan et al., 2013]. Several other factors are able to modulate the expression of 11β-HSD2 such as pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), nitric oxide, oxygen, prostaglandin and PPARδ agonists [Chisaka et al., 2005; Kossintseva et al., 2006; Sun et al., 1997; Alfaidy et al., 2002; Homan et al., 2006; Hardy et al., 1999; Julan et al., 2005].

In addition to its role as a limiting factor of glucocorticoid access to the fetal circulation, 11β-HSD2 might also regulate the local steroid action within the placenta itself. Driver et al. [2001, 2003] detected expression of a functional MR and corticosteroid-induced expression of SGK via MR in trophoblast cells. Therefore, placental 11β-HSD2 may limit the activation of MR by cortisol like it does in

mineralocorticoid-target tissue.

1.1.6 ABC Transporter

The ATP-binding cassette (ABC) transporter molecules phospho-glycoprotein (P-gp, a synonym is multidrug resistance protein 1 (MDR1)) and breast cancer resistance protein (BCRP) were discovered by Kartner et al. [1983] and Doyle et al. [1998] and they are shown to be involved in the exportation of substrates including cortisol molecules back into the maternal circulation [Hahnova-Cygalova et al., 2011; Iqbal et al., 2012]. P-gp and BCRP are encoded by the genes ABCB1 and ABCG2, respectively, and both are expressed in several tissues such as kidney, liver, pancreas, colon, lungs and placenta and they function as drug transporter molecules [Thiebaut et al., 1987; Maliepaard et al., 2001]. In the placenta, both transporter molecules are expressed in the syncytiotrophoblast layer [Kolwankar et al., 2005; Sun et al., 2006]. During pregnancy, expression of P-gp decreases towards term, whereas BCRP expression does not change [Gil et al., 2005; Mathias et al., 2005]. In the placenta, they protect the fetus against xenobiotics such as chemotherapeutics and antibiotics, but also against various endogenous compounds [Zhou, 2008; Polgar et al., 2008]. Mares-Sámano et al. [2009] showed *in silico* that steroids can bind to both P-gp and BCRP and van Kalken et al. [1993] showed that P-gp can export cortisol which suggests that these transporter molecules are parts of the placental glucocorticoid barrier. As the transporter molecules modulate the cellular availability of cortisol, they are involved in the cortisol action inside of cells. In placenta-related diseases such as IUGR, infection and in pre-term labor, the expression of P-gp and BCRP seems to be altered [Evseenko et al., 2007; Mason et al., 2011; Meyer zu Schwabedissen et al., 2006].

Figure 1.8 shows the glucocortcoid barrier and molecules involved in the local glucocorticoid action in the placenta.

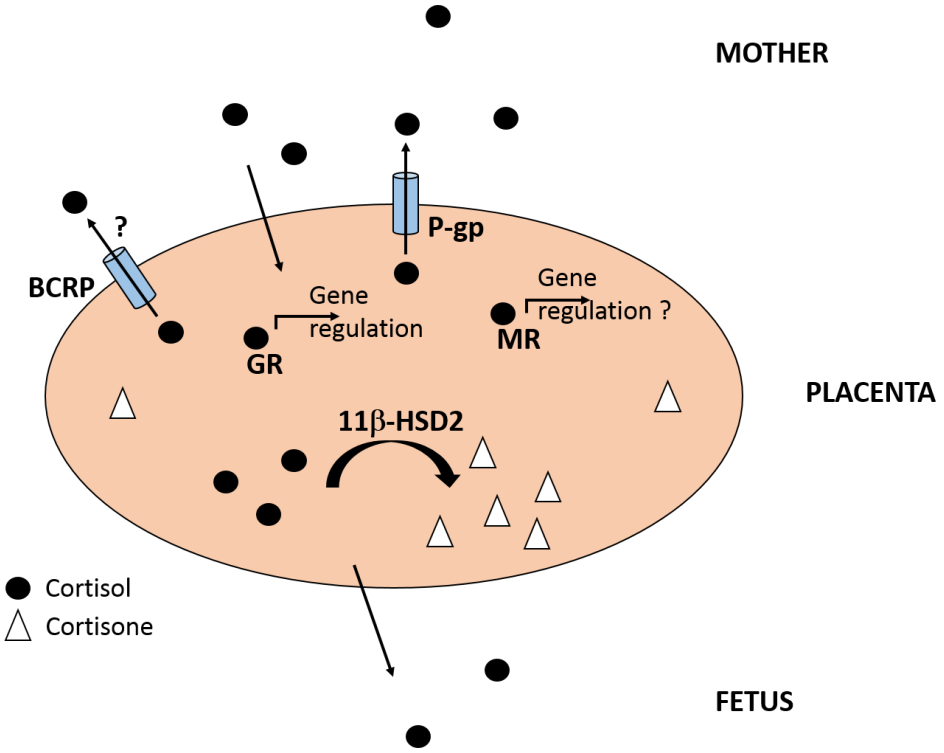

Figure 1.8: **Diagram of the GC barrier and GC local action in the placenta.** Molecules involved in the GC local action in the placenta are the GC receptors GR and MR as well as transporter molecules P-gp and BCRP which export cortisol back into the maternal circulation. The enzyme 11β-HSD2 fulfills the role of the glucocorticoid barrier in the placenta by converting active cortisol into cortisone.

1.2 Human Placenta

The human placenta is the interface between the mother and the fetus, fulfilling important roles such as nutrient transfer; hormone, cytokine, and signalling molecule production; protection of the fetus against the maternal immune system and pathogens; and other barrier functions. The placenta develops from the trophoblast, whereas the embryoblast develops into the fetus. Important during the trophoblast development is the differentiation of trophoblast cells into the fused syncytium which is the site of feto-maternal exchange as well as into extravillous trophoblast which are important for a successfull invasion into maternal tissue [Tarrade et al., 2001]. This chapter describes the morphology, differentiation and functions of the placenta.

1.2.1 Placental Morphology

The placenta consists of several different components such as the chorionic plate, which is the surface of the placenta facing the fetus, the villous trees with the intervillous space, and the basal plate, which is the side of the placenta facing the maternal tissue (Figure 1.9) (Baergen [2011] and Strauss [1964]). The term placenta can be separated into 10-40 so-called cotyledons, consisting of villous trees. The villous tree itself consists of syncytiotrophoblast (ST), cytotrophoblast cells (CT), connective tissue (= mesenchymal stromal cells), and fetal capillaries and veins and its development is described in chapter 1.2.2. Between the villous trees is the intervillous space, which is bathed with maternal blood. The extravillous trophoblast cells (EVT) play an important role in the remodeling of the maternal decidual vessels, which is described in chapter 1.2.4. The contact side of the maternal circulation with fetal tissue is the ST and its differentiation is described in chapter 1.2.5. The structure of the placenta with its close contact of maternal and fetal circulation allows the successful exchange of nutrients and waste between mother and fetus.

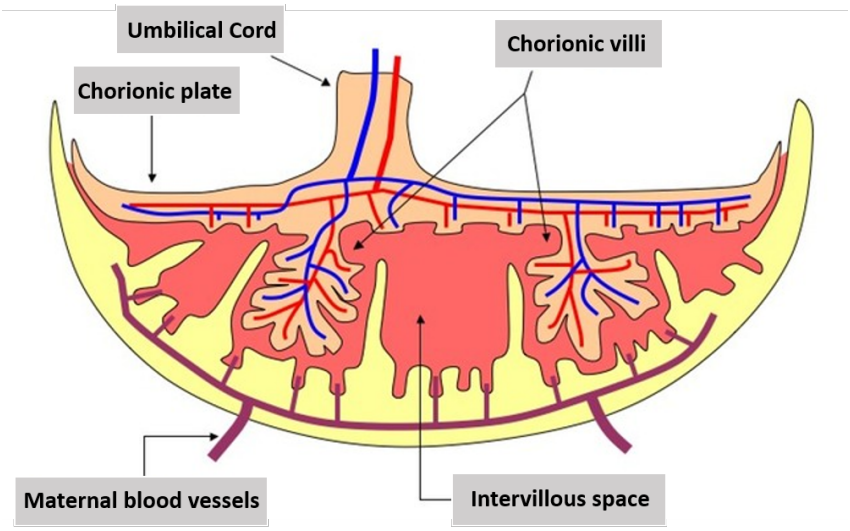

Figure 1.9: **Diagram of placenta.** Cross section of the human placenta. The chorionic plate is facing the fetus. The chorionic villi are covered by the syncytiotrophoblast which is in direct contact with the maternal blood. Adapted from: http://www.ib.bioninja.com.au/higher-level/topic-11-human-health-and/114-reproduction.html.

1.2.2 Villous Tree Development

After fertilization, the zygote divides and thereby develops into a blastocyst. This blastocyst reaches the uterine cavity on day 6-7 post conception (p.c.) and the implantation process begins [Aplin, 2000]. The blastocyst attaches at the endometrium with the inner cell mass (ICM) oriented to that side. The ICM develops into the embryo, whereas the trophoblast part of the blastocyste develops into the placenta. The trophoblastic cells invade the endometrium and start dividing, thereby forming an inner layer, the cytotrophoblast, and an outer layer, the syncytiotrophoblast. The maternal facing syncytiotrophoblast is generated by fusion of cytotrophoblast cells (see chapter 1.2.5). On day 8 p.c., vacuoles, called lacunae, develop within the syncytiotrophoblast [Baergen, 2011]. Between day 8 and 12 p.c., the syncytiotrophoblast expands and then covers the entire blastocyst. This stage, day 8-12 p.c., is called the lacunar phase. After that, the transformation from the lacunar system to the villous system occurs between day 13-28 p.c. Cytotrophoblast cells invade the areas between the lacunae, called trabeculae, from the chorionic plate to the maternal side [Baergen, 2011] and each of the invaded trabeculae will develop into one villous tree. These primary villi consist of syncytiotrophoblast and cytotrophoblast

cells. During the transformation to secondary villi, embryonic mesenchymal cells, which cover the inner surface of the blastocyst cavity, invade the villi [Castellucci et al., 1990]. Between day 18-20 p.c., some of the mesenchymal cells differentiate into hemangioblastic progenitor cells, which then develop into fetal capillaries [Dempsey, 1972]. Presence of these fetal capillaries within the villous stroma marks the transition into tertiary villi. Hence, the processes involved in the villous tree development are trophoblastic sprouting, mesenchymal invasion and local fetal angiogenesis. Beginning with the second month of pregnancy, the mesenchymal tertiary villi undergo differentiation and different villous types occur: immature intermediate villi, mature intermediate villi, stem villi, and terminal villi [Huppertz, 2008].

1.2.3 Placental Vascularization

As mentioned above, placental vascularization starts with the development of the tertiary villi: extra-embryonic mesenchymal cells differentiate into hemangiogenic progenitor cells [Demir et al., 2007]. This process, taking place from day 21-32 p.c., is a vasculogenesis process leading to new vessels formation. Hematopoietic cells, which are precursors of blood cells, and angioblastic cells, which later differentiate into endothelial cells, evolve from differentiation of the hemangiogenic progenitor cells. From day 32 p.c. until term, angiogenesis, which is the connection of preformed vessels with each other, is observed [Kaufmann et al., 2004]. Several growths factors such as vascular endothelial growth factor (VEGF), placental growth factor (PlGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and angiopoietins are involved in vasculogenesis and angiogenesis. In the beginning of vascularization, villous trophoblast cells secrete VEGF, PlGF, and FGF, whereas in later stages, the newly occurred smooth muscle cells produce VEGF, FGF, and angiopoietins [Sharkey et al., 1993; Vuorela et al., 1997; Riddell et al., 2012]. After midgestation, length of vessels and their volume grow exponentially to meet the demands of the fetus.

1.2.4 Extravillous Trophoblast Differentiation

Also during early stages of the placentation, at the basal plate, which is the contact side of syncytiotrophoblast and maternal tissue, extravillous trophoblast cells (EVT) deeply invade the endometrium (Figure 1.10). In the beginning, the syncytiotrophoblast is in contact with the endometrium, but after cytotrophoblast cells

have reached the basal plate, the syncytiotrophoblast is disrupted at some locations. In these areas, cytotrophoblast cells differentiate into the proliferative EVTs, which are the stem cells of the EVTs [Bulmer et al., 1988; Genbacev et al., 1993]. This cell population can either differentiate into interstitial EVTs, which infiltrate the uterine wall, or into endovascular EVTs, which invade maternal spiral arteries. During the invasion process, the endovascular EVTs infiltrate the vessel walls and replace the endothelium and as a result the decidual vessels are converted into uteroplacental vessels characterized by low resistance which increases the blood flow [Pijnenborg et al., 1980; Thaler et al., 1990]. Fibrinoid is deposited, possibly by endovascular and interstitial trophoblasts, in the spiral arteries [Pijnenborg et al., 2006].

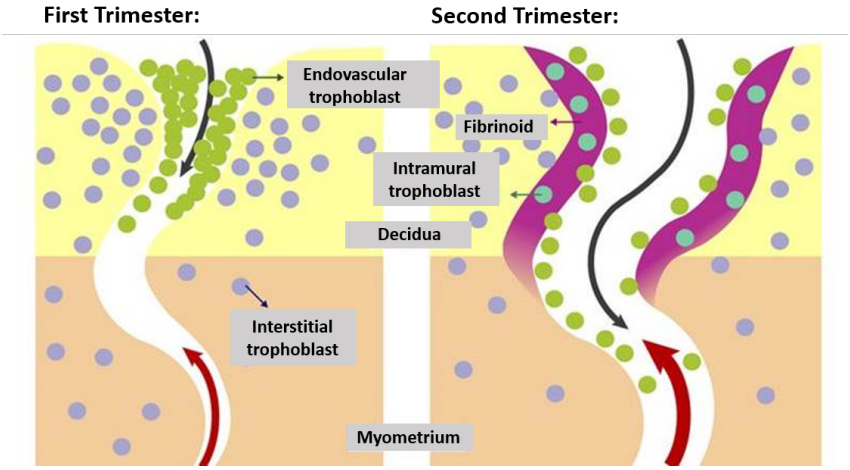

Figure 1.10: **Remodelling of the uterine spiral arteries by extravillous trophoblast.** The extravillous trophoblast cells invade the myometrium to remodel the uterine spiral arteries into low resistance vessels. Black arrow = movement of EVTs, red arrow = blood flow. Adapted from Pijnenborg et al. [2006].

1.2.5 Syncytiotrophoblast Differentiation

The syncytiotrophoblast (ST) is the direct contact side between the mother and the fetus. It is a multinucleated cell layer and it arises from fusion of underlying cytotrophoblast cells. The membrane facing the fetal side is called basal membrane (BM) and the membrane facing the maternal side is called microvillous membrane (MVM). Fusion of cytotrophoblast cells by differentiating into ST and shedding of syncytial knots at the MVM are parts of the normal trophoblast cell turnover (Figure 1.11) [Huppertz et al., 1998].

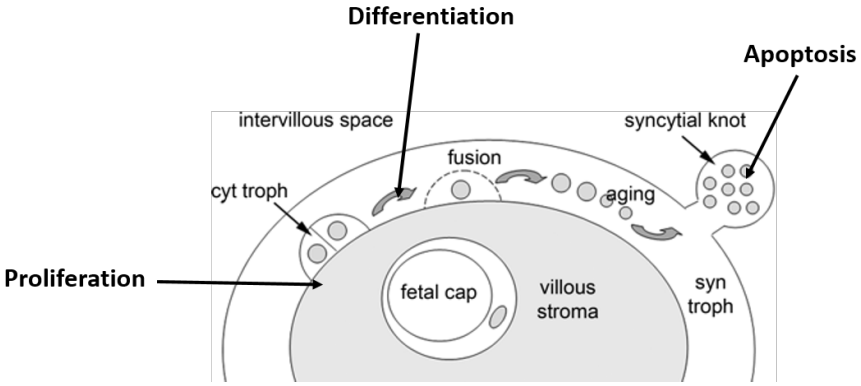

Figure 1.11: **Trophoblast turnover.** Cytotrophoblast cells (= cyt troph) proliferate, then undergo differentiation and fuse with the syncytiotrophoblast (= syn troph). Nuclei are accumulated in syncytial knots which are shed from the layer while undergoing apoptosis. Adapted from Carter [2008].

1.2.5.1 Formation of ST

The syncytiotrophoblast (ST) develops within the first days of implantation of the blastocyst into the decidua (Figure 1.12). Notably, implantation does not take place without the ST since this cell layer has penetrating capacity. The development of this first ST is characterized by cell-cell-fusion of cytotrophoblast cells [Pötgens et al., 2002]. The function of the ST changes over time and the penetrating capacity is lost, instead the ST develops important transport and endocrine functions. After the first period, the ST grows and is maintained by fusion of cytotrophblasts with the ST layer. The cytotrophoblast cells undergo a differentiation process before they eventually fuse with the ST [McKenzie et al., 1998]. This incorporation of cytotrophoblasts at the BM takes place throughout pregnancy and in order to maintain ST homeostasis, material of the ST needs to be removed at the MVM. The nuclei remain in the ST for around 3-4 weeks before they enrich in certain areas of the MVM as syncytial knots [Jones and Fox, 1977], which are shed into the maternal capillary system.

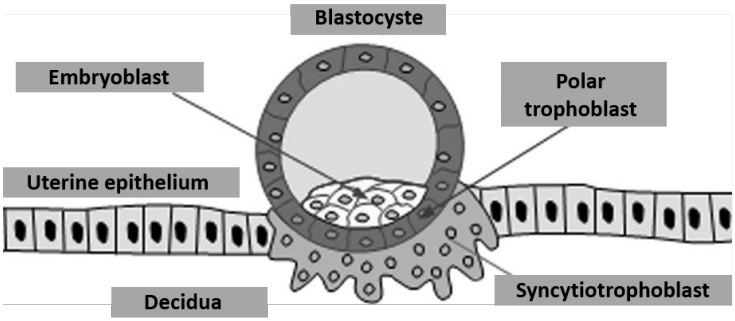

Figure 1.12: **Blastocyst implantation with early syncytiotrophoblast.** Adapted from Huppertz [2008]

1.2.5.2 Characteristics of ST

The syncytiotrophoblast is a multinucleated cell layer covering the whole villous tree system of the placenta. This layer is in a postmitotic state [Burton and Jones, 2009]. Furthermore, the ST shows only a low transcriptional activity demonstrated with different methods (staining for active Pol-II and histone modifications, nucleoside incorporation assay) by Ellery et al. [2009]. Specifically, the nuclei within the ST layer are at different ages and therefore differently active. Some of the nuclei display transcriptional activity whereas other nuclei do not have this capacity anymore [Ellery et al., 2009]. The size of the ST surface area grows from 0.8 m^2 at week 12 of gestation to 12-14 m^2 at term [Boyd, 1984]. The ratio of nuclei of ST to a cytotrophoblast nucleus remains constant throughout pregnancy and is about 9 [Simpson et al., 1992].

1.2.5.3 Molecules involved in ST Formation

Regulation of cytotrophoblast fusion is diverse and several factors and signalling pathways are involved. For example, different factors such as growth factors (epidermal growth factor (EGF), vascular endothelial growth factor (VEGF)), stimulating factors (colony-stimulating factor (CSF), granulocyte-macrophage-CSF (GM-CSF)), and hormones (human chorionic gonadotropin (hCG)) can promote the syncytialization process, whereas other molecules such as tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) inhibit the syncytium formation [Morrish et al., 1987; Crocker et al., 2001; Garcia-Lloret et al., 1994; Shi et al., 1993; Leisser et al., 2006; Morrish et al., 1991].

Signaling pathways involved in the fusion process of cytotrophoblasts are the MAPK pathways (namely the p38 and the $ERK_{1/2}$ MAPK) which are shown to promote syncytial fusion [Daoud et al., 2005; Delidaki et al., 2011] and the PKA pathway regulating important factors such as Syncytin-1 which participates as a fusogene in the syncytial fusion process [Knerr et al., 2005].

In addition to the fusogenes Syncytin-1 and Syncytin-2 (see below), also other molecules such as galectin-3 and its receptor CD98, placental protein 13 (PP13), connexin 43, caspase-8, and members of the ADAM (a disintegrin and a metalloproteinase domain) family are shown to be involved in the trophoblast fusion or are candidate molecules which might play a role in this process [Kudo et al., 2003b; Dalton et al., 2007; Than et al., 2004; Frendo et al., 2003; Black et al., 2004; Huovila et al., 1996].

Syncytin-1 and Syncytin-2

Syncytin-1 was discovered in 1999 and it is the envelope protein of the human endogenous retrovirus-W (HERVW) [Blond et al., 1999; Mi et al., 2000]. Syncytin-1 is a membrane glycoprotein and has fusogenic capacity when binding to its receptor, the RD114/mammalian type D retrovirus receptor (RDR, synonyms are ASCT2 and ATB0) [Blond et al., 2000]. Frendo et al. [2003] showed that Syncytin-1 is directly involved in fusion of trophoblasts. In other tissues, the promotor of Syncytin-1 is methylated preventing its expression [Matousková et al., 2006].

In 2003 Syncytin-2, envelope protein of the HERV-FRD gene, was discovered [Blaise et al., 2003]. It is expressed in the placenta and binds to the receptor MFSD2 (major facilitator superfamily domain containing 2) [Malassiné et al., 2007; Esnault et al., 2008]. Syncytin-2 is involved in the fusion process of trophoblast cells and also possesses immunosuppressive activity [Vargas et al., 2009; Mangeney et al., 2007].

Other HERV envelope proteins such as EnvP(b) and EnvV are expressed in the placenta, but they do not play a role in the fusion process [Vargas et al., 2012].

1.2.6 Placenta and Apoptosis

1.2.6.1 General Description of Apoptosis

Apoptosis is the process of programmed cell death and the term "'apoptosis"' was first used by Kerr et al. [1972]. In contrast to necrosis, which is an unregulated cell death process involving several cells (for example induced by tissue damage), apoptosis is an energy-dependent and controlled process affecting single cells [Kerr et al., 1972]. Apoptosis is of fundamental importance for the development of organisms, for the maintenance of cell homeostasis and for the regulation of the immune system. However, excess or too little apoptosis is involved in pathologies such as autoimmune and neurodegenerative diseases and cancer [Elmore, 2007].

During apoptosis, at the cellular level, the apoptotic cell seperates from neighbouring cells, cytoplasm and nucleus condenses and apoptotic bodies detach from the cell and these are being phagocytosed by macrophages and other cells [Kerr et al., 1972]. At the molecular level, many proteins are cleaved by enzymes (caspases) and the DNA is fragmented by nucleases. The activation of the caspases is triggered by two different pathways, the extrinsic and intrinsic pathway (Figure 1.13), which are described below.

Extrinsic and Intrinsic Pathway

The transmembrane death receptors, which belong to the TNF receptor superfamily, play a central role in the activation of the extrinsic pathway [Ashkenazi and Dixit, 1998] (Figure 1.13). After ligand binding, the death receptors such as CD95 (=Fas) and TNFR1 oligomerize and intracellular adapter molecules such as FADD and TRADD bind to the receptors [Chinnaiyan et al., 1995; Hsu et al., 1995]. Death domains of the receptors and the adapter molecules are important for their interaction. The procaspase-8 is being recruited to this DISC (=death-inducing signalling complex) complex where it is cleaved to active caspase-8 [Kischkel et al., 1995].

Members of the Bcl-2 (=B-cell lymphoma-2) family such as Bcl-2, Bcl-X_L and Bax play a central role in the activation of the intrinsic pathway [Leibowitz and Yu, 2010] (Figure 1.13). These molecules can either act anti-apoptotic or pro-apoptotic and they can be divided in three groups [Leibowitz and Yu, 2010]. Members of the first group such as Bcl-2 and Bcl-X_L are characterized by 4 BH (=Bcl-2 homology) domains and they are anti-apoptotic. Members of the second group such as Bax and Bak possess 3 BH domains and they are pro-apoptotic. Members of the BH3-only group such as Bad and Bid are involved in the detection of apoptotic signals. The key function of the Bcl-2 members is the regulation of the mitochondrial pore (mitochondrial permeability transition pore = MPTP) formation [Vander Heiden and Thompson, 1999; Crompton, 1999]. Homo- and heterooligomerization processes of the Bcl-2 members are important for the pore formation and so the ratio of pro- and anti-apoptotic Bcl-2 members is involved in the decision whether a cell becomes apoptotic [Oltvai et al., 1993; Yin et al., 1994; Chittenden et al., 1995]. The formation of the MPTP leads to destruction of the mitochondrial membrane potential and to the release of pro-apoptotic factors and cytochrom-c from the intermembrane space of the mitochondria into the cytosol [Vayssiere et al., 1994; Bossy-Wetzel et al., 1998; van Loo et al., 2002]. In the presence of ATP, a complex called apoptosome consisting of cytochrom-c molecules and apaf-1 (= apoptotic protease activating factor-1) molecules is formed and the procaspase-9 is actived [Hill et al., 2004; Yu et al., 2005].

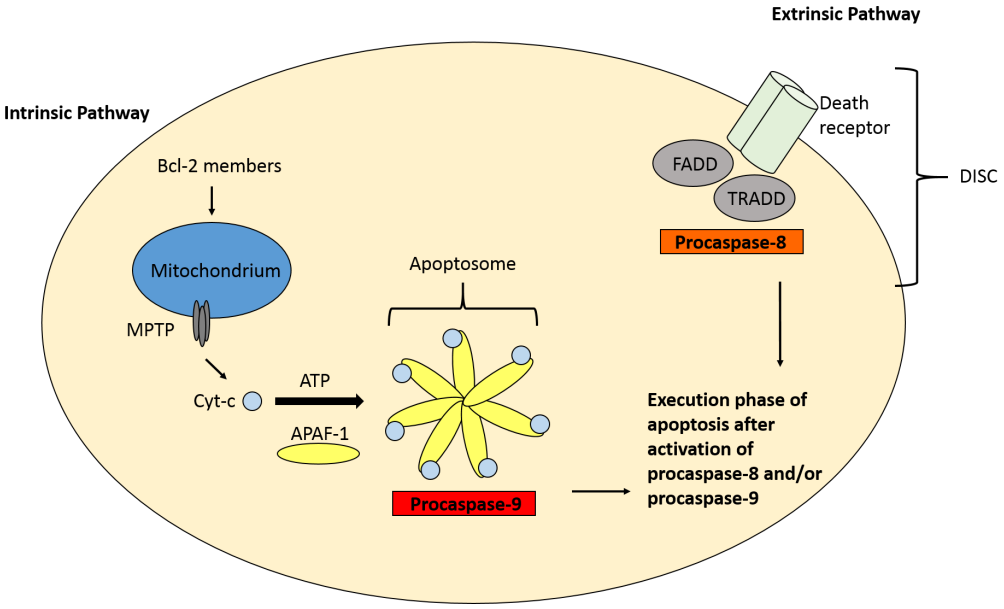

Figure 1.13: **Extrinsic and Intrinsic Pathway of Apoptosis.** Apoptosis can be activated by the intrinsic or extrinsic pathway. **(Left)** Intrinsic pathway: Members of the Bcl-2 family sense death stimuli and are involved in the formation of the mitochondrial pore (= MPTP) and in the release of cytochrom-c (= Cyt-c). In the cytoplasm, the apoptosome is formed from Apaf-1 and Cyt-c molecules in the presence of ATP. This complex activates the procaspase-9. **(Right)** Extrinsic pathway: Death receptors oligomerize after ligand binding and adapter molecules such as FADD and TRADD bind via death domains to the receptors. This DISC complex activates the procaspase-8. Activation of the initiator caspases-8 and -9 lead to activation of the execution phase of apoptosis.

Execution Phase of Apoptosis

Both actived (cleaved) caspase-8 and caspase-9 initiate the execution phase of the apoptotic process which starts with the activation (cleavage) of the effector caspases (e.g. caspase-3/7). Caspases (= cystein-dependent aspartate specific protease) belong to the family of the ICE/CED-3 cysteinproteases [Alnemri et al., 1996]. Caspases are synthezised as procaspases which require to be cleaved to unfold their catalytic activity [Orth et al., 1996]. All caspases cleave their substrates after the amino acid aspartate within a recognizing sequence, for example, caspase-3 and -7 cleave their substrates at the DEVD recognition sequence [Thornberry et al., 1997; Nicholson, 1999]. Many morphological features of apoptosis can be explained by the cleavage of the substrates from the effector caspases. PARP (= poly-(ADP-ribose)-polymerase) and the inhibitor of the endonuclease CAD (= caspase-activated DNase) are among the estimated 200 substrates of the caspases [Sakahira et al., 1998; Nicholson, 1999].

1.2.6.2 Apoptosis in Placenta during Pregnancy

Apoptosis takes place in normal placental cell turnover, which includes proliferation of cytotrophoblast cells, their fusion into the syncytiotrophoblast layer and the apoptotic event of syncytial shedding. This cycle guarantees maintenance of the syncytiotrophoblast by balancing proliferation and apoptosis of trophoblast cells. During the lifetime of a placenta, apoptotic processes are observed in placental tissue and the frequency of apoptosis rises towards the end of pregnancy [Smith et al., 1997b; Gruslin et al., 2001; Athapathu et al., 2003]. Interestingly, apoptotic processes in the placenta are elevated throughout pregnancy in placental diseases [Sharp et al., 2010].

1.2.6.3 Apoptosis during ST Differentiation

In addition to its role in placental cell turnover, apoptotic features develop during cytotrophoblast differentiation into the syncytiotrophoblast layer. Hallmarks of apoptosis such as the phosphatidylserine (PS)-flip, activation of caspase-8 and changes in the expression of pro- and anti-apoptotic molecules (Chapter 1.2.6.1) are detected in the placenta during the differentiation process.

During early apoptosis, PS is externalized from the inner to the outer leaflet of the cell membrane. Lyden et al., 1993, showed that the PS-flip arises during differentiation of BeWo cells (a placental cell line) and Huppertz et al., 1998, demonstrated that the PS-flip also occurs in primary placental tissue. Furthermore, the

PS-flip seems to be a prerequisite for the fusion process since PS-antibodies can inhibit fusion (Adler et al., 1995).

In apoptosis, activation of caspase-8, an initiator caspase, is an early event that results from activation of the extrinsic apoptotic pathway. Several studies revealed that activated caspase-8 is primarily localized in cytotrophoblast cells (De Falco et al., 2004, Gauster et al., 2009), suggesting that its presence is required for the initiation of the differentiation process. Black et al., 2004, showed that inhibiting caspase-8 function by antisense oligonucleotides or by a peptide inhibitor impairs the fusion process, which supports the above mentioned hypothesis. Another interesting aspect is that several caspases seem to be involved in differentiation processes of different cell types. For example, caspase-3 plays a role in the differentiation of keratinocytes (Weil et al., 1999) and caspase-8 is involved in the monocyte maturation (Secchiero et al., 2002). These results suggest the possibility that caspases have a non-apoptotic function in addition to their known role in the apoptotic process.

After cytotrophoblast cell fusion, the apoptotic cascade is stopped and no further signs of apoptosis develop aside from the syncytial shedding. Pro- and anti-apoptotic molecules belong to the Bcl-2 protein family, which are expressed in trophoblast cells. The syncytiotrophoblast layer highly expresses Bcl-2, an anti-apoptotic protein, and moreover, in a placental cell culture model, Bcl-2 expression was increased after differentiation (Sakuragi et al., 1994). These observations suggest that up-regulation of Bcl-2 might be a mechanism in placental cells to end the apoptotic events.

1.2.7 Functions of the Placenta

1.2.7.1 Placental Nutrient Transport

One role of the placenta is to ensure sufficient nutrient transfer to the fetus. For the majority of nutrients required by the fetus, the maternal circulation is the only source. Some molecules like oxygen can pass through the placental membrane, but others need to be transported by specific transport proteins. Thus, the placental membrane expresses several transport proteins for glucose, amino acids and fatty acids (Figure 1.14). Specifically, the syncytiotrophoblast is the primary barrier and both the maternal facing membrane, the microvillous membrane (MVM), and the fetal facing basal membrane (BM) express these transporters. Maternal malnutrition or deregulation of transporter molecules can compromise fetal development by leading to abnormal fetal growth [Lager and Powell, 2012].

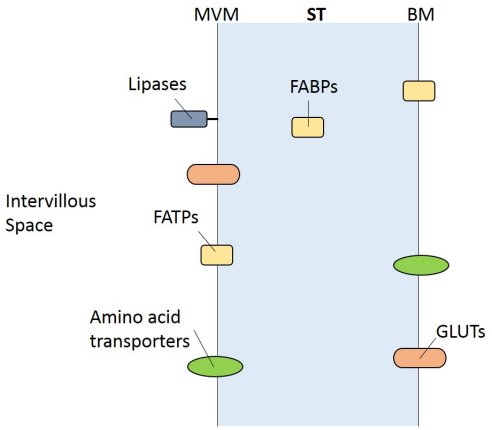

Figure 1.14: **Transport in ST.** Several different transporter molecules for glucose, amino acid and fatty acid transport are expressed in the MVM (= microvillous membrane) and BM (= basal membrane) of the syncytiotrophoblast. FABP = fatty acid binding protein, FATP = fatty acid transport protein, GLUT = glucose transporter. Adapted from Lager and Powell [2012].

Glucose Transport

As the fetus does not produce glucose [Kalhan and Parimi, 2000], the maternal circulation is the source for glucose transfer from mother to fetus. Transport of glucose is mediated via glucose transporters (GLUTs), of which GLUT1 is the main glucose transporter in the syncytiotrophoblast [Jansson et al., 1993]. Glucose transport is a facilitated diffusion process [Johnson and Smith, 1980], as it is dependent on the glucose concentration gradient between mother, placenta, and fetus. Higher levels of GLUT1 are expressed on the maternal-facing membrane of the syncytiotrophoblast than on the membrane facing the fetus [Jansson et al., 1993]. The placenta itself has a high demand in glucose for its metabolism (0.13-0.33 mmol/min/kg); around one-third of the glucose taken up by the placenta is metabolized via glycolysis, the pentose phosphate pathway, and non-triose phosphate pathways in the placenta [Hay, 1995; Lager and Powell, 2012]. The fetus has a glucose consumption of 0.07 mmol/min/kg and needs glucose as the primary substrate for growth.

Amino Acid Transport

Non-essential amino acids can be synthesized by the fetus, but essential amino acids need to be transported from the maternal circulation to the fetus passing through the placenta. As the amino acid concentration is higher in the fetal than in the maternal circulation [Philipps et al., 1978], the transport of amino acids is active, specifically it is a secondary active transport. Amino acid transporters in the placenta belong to sodium-coupled transporters, exchangers, and non-exchange efflux transporters and more than 20 different of these transporters are expressed in the placenta [Jansson, 2001]. The uptake of amino acids can be regulated by cytokines and hormones including insulin, IGF1, and leptin [Jansson et al., 2003]. In addition to the transfer of amino acids, the placenta can also metabolize amino acids to produce new amino acids, other compounds or energy as well as use amino acids for protein synthesis. IUGR fetuses show a lower amino acid concentration in umbilical cord blood than normal fetuses and exhibit an impaired placental amino acid transport system [Cetin et al., 1988; Jansson, 2001].

Fatty Acid Transport

Fatty acids derive from triglycerides, which are hydrolyzed at the maternal surface of the MVM by lipoprotein lipases [Waterman et al., 1998; Lindegaard et al., 2005]. These fatty acids are then transported in the placenta by fatty acid transport proteins (FATPs), of which FATP1 and 4 are expressed in the placenta [Larqué et al., 2006; Kazantzis and Stahl, 2012]. Fatty acid binding proteins (FABPs) traffic fatty acids within the syncytiotrophoblast and several subtypes of FABPs are expressed in the placenta [Campbell et al., 1998]. The fatty acids are either transported to the fetus or are metabolized in the placenta by esterification or beta-oxidation [Ramsay et al., 1991; Oey et al., 2006]. Clinically, IUGR placentae show a dysregulated expression of the lipoprotein lipase [Tabano et al., 2006; Gauster et al., 2007].

1.2.7.2 Placental Endocrine Function

Another role of the placenta is to produce several different hormones, specifically the syncytiotrophoblast layer fulfills the endocrine function of the placenta. The hormones are involved in many processes such as the establishment and maintenance of pregnancy, regulation of vascular development, adaptation and regulation of maternal metabolism to pregnancy, regulation of fetal growth and parturition,

and placental adaptation to adverse environments.

The hormones human chorionic gonadotropin (hCG), progesterone (P4) and estradiol (E2) are described in more detail below. Growth factor hormones such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) are involved in the regulation of vascular development [Burton et al., 2009]. Human placental lactogen (hPL) acts as a growth hormone in the placenta, i.e. hPL stimulates maternal IGF production and it leads to a higher availability of amino acids and glucose [Handwerger and Freemark, 2000]. Moreover, adipocytokines and insulin-like growth factors (IGFs) regulate maternal metabolism as well as placental and fetal growth [D'Ippolito et al., 2012; Hiden et al., 2009]. Leptin seems to be involved in many regulating processes such as placental growth, angiogenesis and immunmodulation as well as fetal organogenesis [Gambino et al., 2010]. Receptors for these hormones are expressed in the neighboring tissues as well as in the placenta itself [Fowden et al., 2015] and therefore, the hormones act in a paracrine as well as autocrine manner suggesting effects both in maternal and fetal compartments (Figure 1.15).

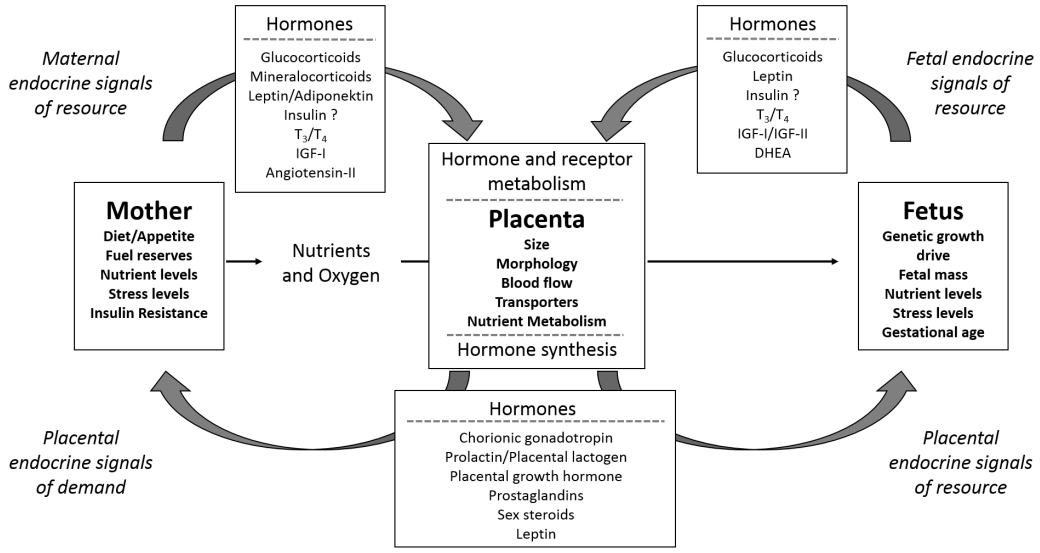

Figure 1.15: **Cross-talk of maternal, fetal and placental hormones and their effects on mother, fetus and placenta.** DHEA = dihydroepiandrostenedione, IGF = insulin-like growth factor, T_3 = tri-iodothyronine, T_4 = thyroxine. Adapted from Fowden et al. [2015].

hCG

hCG is a peptide hormone, which is secreted by the syncytiotrophoblast. It consists of two subunits, an α- and a β-subunit, of which the β-subunit is specific for hCG. The α-subunit is also a subunit of the luteinizing hormone (LH), the follicle-stimulating hormone (FSH), and the thyroid-stimulating hormone (TSH). Landefeld et al. [1976] identified in first trimester placentae the mRNA which encodes for the α-subunit precursor, Daniels-McQueen et al. [1978] isolated both hCG subunit mRNAs, and in 1994 the crystal structure of hCG was revealed by Lapthorn et al. [1994]. hCG and LH bind to the same hCG/LH receptor belonging to the GPCR family [McFarland et al., 1989; Minegishi et al., 1990; Jia et al., 1991] and this receptor is expressed by several tissues including myometrium, endometrium, placenta, fetal membranes and decidua [Reshef et al., 1990].

The hCG concentration rises during pregnancy, reaching a maximum between week 9 and 10 of gestation, and then the concentration decreases and stays at a low constant level until the end of the pregnancy [Cole, 2010]. As the high hCG concentrations are characteristic for a pregnancy, detection of hCG is used in pregnancy tests [Chard, 1992].

hCG has multiple functions on the fetoplacental unit. One very important function of hCG is the stimulation of the corpus lutuem to produce progesterone during the first seven to eight weeks of pregnancy [Schmitt et al., 1996]. Further, hCG appears to be involved in implantation as several effects of hCG on the endometrium such as upregulation of LIF, VEGF and MMP-9 could be demonstrated [Licht et al., 2007]. Also, hCG is involved in the control of endometrial vascularization and placentation as it can stimulate migration of endothelial cells as well as induce neovascularization [Zygmunt et al., 2002]. Another role of hCG is the suppression of an immune response against fetoplacental tissue by upregulating MIF (= macrophage inhibitory factor) in endometrial stromal cells and by attracting regulatory T cells to the uterus [Akoum et al., 2005; Schumacher et al., 2009]. hCG is further involved in modulating the differentiation of cytotrophoblasts into syncytiotrophoblast and the relaxation of myometrial contractions [Eta et al., 1994; Shi et al., 1993]. Many of the mentioned processes are especially important during early pregnancy which might explain the high expression of hCG during the first trimester of pregnancy.

In addition to hCG, which is produced by the syncytiotrophoblast, three other forms of hCG exist, namely the hyperglycosylated hCG produced by cytotrophoblasts, the sulphated hCG produced by the pituitary and the free β-subunit produced by several non-trophoblastic malignancies [Cole, 2012].

Progesterone

During the first seven to eight weeks of pregnancy, progesterone (P4) is secreted by the corpus luteum of the ovary gland. Then the so-called luteo-placental shift occurs and the P4 production is taken over by the placenta [Csapo, 1969].

Progesterone belongs to the steroid hormones and binds to nuclear receptors, the progesterone receptors PR-A and -B, which are transcribed from the same gene with alternate promoter usage, as well as to membrane-bound progesterone receptors (mPRs) [Li et al., 2004]. P4 exerts effects via genomic actions through PR-A and PR-B receptors, which act as nuclear transcription factors after hormone binding whereby PR-A can repress the function of PR-B [Vegeto et al., 1993]. Additionally, progesterone also exerts effects via non-genomic actions mediated by either binding to PR-A and -B or to the mPRs [Kowalik et al., 2013]. PRs are expressed in the human uterus [Mote et al., 1999] and in the placenta [Rossmanith et al., 1997; Shanker and Rao, 1999]. Other isoforms of the PR and a changed ratio of PR-A to PR-B might play roles in P4 withdrawal (see below) [Goldman and Shalev, 2007]. The mPRs are expressed in gestational tissues including the endometrium, myometrium and placenta [Fernandes et al., 2005].

The secretion of progesterone is gradually rising towards the end of pregnancy [Albrecht and Pepe, 1990]. Functions of progesterone are important during all phases of gestation. Even before that, progesterone prepares the uterus for implantation during the implantation window to establish uterine receptivity [Li et al., 2011b]. During gestation, it is important for maintaining a successful pregnancy by preventing uterine contractions [Ruddock et al., 2008]. Furthermore, progesterone has a regulatory function as it prevents excessive trophoblast migration by negatively regulating MMPs (matrix metalloproteinases) which are important in the extravillous trophoblast invasion process [Higuchi et al., 1995]. Also, P4 exerts immunomodulatory functions during pregnancy. For example, P4 can modulate cytokine production by macrophages and T cells shifting the cytokine production to a Th2 response [Piccinni et al., 1995]. Near term, it is hypothesized that a functional P4 withdrawal occurs to induce labour. In subprimate mammals, a progesterone withdrawal leads to the onset of labour [Zakar and Hertelendy, 2007]. In contrast, in humans, the progesterone concentration does not decline at the end of pregnancy, but the expression of the PRs (altered ratio of PR-A to PR-B and possibly involvement of PR-C) might explain a functional withdrawal [Merlino et al., 2007]. Progesterone is used as a therapeutic application in pregnancies with a risk of abor-

tion, miscarriage and pre-term labour [Di Renzo et al., 2005].

Estrogen

Estradiol (E2) like progesterone is a steroid hormone, but the placenta is unable to directly synthesize estradiol from progesterone because the enzyme cytochrome P450 17α-hydroxylase is not expressed. Instead the placenta requires DHEA or DHEAS from maternal and fetal circulation which then is used as a precursor molecule for E2 production [Baulieu and Dray, 1963; Siiteri and MacDonald, 1966; Branchaud et al., 1983].

E2 binds to two nuclear receptors, ERα and ERβ, as well as to a membrane-bound receptor GPR30 [Björnström and Sjöberg, 2005; Prossnitz et al., 2008]. The nuclear receptors are expressed in the placenta [Bukovsky et al., 2003]. After ligand binding, they translocate to the nucleus and bind to ERE sequences which leads to gene expression regulation. The GPR30 is expressed in the endometrium and decidua and might play a role in the implantation process [Kolkova et al., 2010]. Through this membrane-bound receptor, signalling pathways are activated which lead to phosphorylation processes.

Plasma E2 concentration during pregnancy is increasing towards term [Albrecht and Pepe, 1990]. E2 fulfills varies functions at the maternal-fetal interface such as regulation of blastocyst development and implantation, regulation of differentiation of cytotrophoblast into syncytiotrophoblast, remodeling of uterine arteries, modulating the release of MIF which is involved in the immune response, modulating the Th1-type and Th2-type cells (inhibition of Th1 cytokine and stimulation of Th2 cytokine production), and regulation of placental hormone production like leptin which is an important factor in placentation and fetal growth [Seshagiri et al., 2009; Cronier et al., 1999; Albrecht and Pepe, 2010; Ietta et al., 2010; Salem, 2004; Gambino et al., 2010].

1.2.8 Immunological Processes during Pregnancy

Cytokines are mediator molecules in inflammatory processes, but in the feto-maternal unit they also play important roles in regulating processes such as uterine receptivity, implantation, placentation, fetal immunotolerance, uterine expansion and preparation for labour. Thus, cytokines not only play roles in inflammation, they are also involved in regulating physiological processes [Blank et al., 2008]. Deregulation of

cytokines is associated with several diseases such as miscarriage, pre-term labor and pre-eclampsia [Orsi and Tribe, 2008].

Cytokines can be divided into two groups: (1) IL-1, IL-2, IL-6, IL-12, IL-15, IL-18, IFN-γ and TNF-α which belong to the pro-inflammatory cytokines involved in Th1 responses (cell-mediated immunity) and (2) IL-4, IL-5, IL-10, IL-13 and GM-CSF (granulocyte-macrophage colony-stimulating factor) which belong to the anti-inflammatory cytokines involved in Th2 responses (humoral immunity) [Challis et al., 2009]. During pregnancy cytokines are produced by the placenta [Bowen et al., 2002]. Pregnancy is characterized by a predominance of Th2 cytokines (= Th2 phenomenon) which is important in preventing aberrant inflammation processes and allograft rejection of the fetus [Wegmann et al., 1993]. Alterations in this Th2 shift are associated with adverse pregnancy outcome and preterm delivery (Figure 1.16).

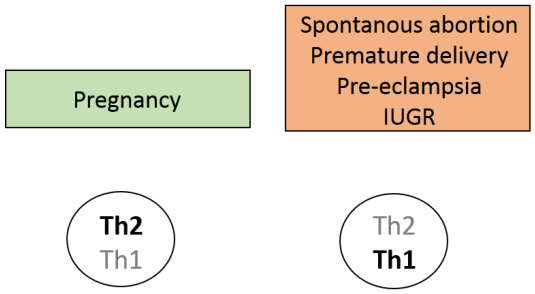

Figure 1.16: **Th2 phenomenon during pregnancy.** Balance of Th1 and Th2 cytokines during pregnancy is shifted towards a predominance of Th2 cytokines. Adapted from Challis et al. [2009].

1.2.8.1 Cytokines involved in Pregnancy-related Processes

In the first trimester of pregnancy, EVTs invade the maternal spiral arteries (Chapter 1.2.4). During this invasion, an extensive remodelling of maternal spiral arteries occurs, a process in which the matrix-metalloproteinases (MMPs) play a key role [Zhou et al., 2003]. Important in this process is the cytokine IL-1β as it increases the trophoblast MMP-9 expression and activity [Librach et al., 1994]. Also, the cytokines TNF-α, IL-1α, and M-CSF (macrophage colony-stimulating factor) increase MMP-9, whereas TGF-β leads to its inhibition [Meisser et al., 1999].

At the end of pregnancy, the cervical ripening and initiation of myometrial contractions during the parturition process are also regulated by cytokines

(Figure 1.17). Sennström et al. [2000] observed an increase in IL-6, IL-8 and G-CSF (granulocyte-CSF) during the cervial ripening process. And IL-1 increases the prostaglandin E_2 (PGE_2) expression in myometrial cells which is important for myometrial contraction [Todd et al., 1996; Olson, 2003]. MMPs are also involved in the parturition process as they are able to remodel the extracellular matrix. The concentration of MMP-9 is elevated in placental and fetal membranes during labour [Xu et al., 2002].

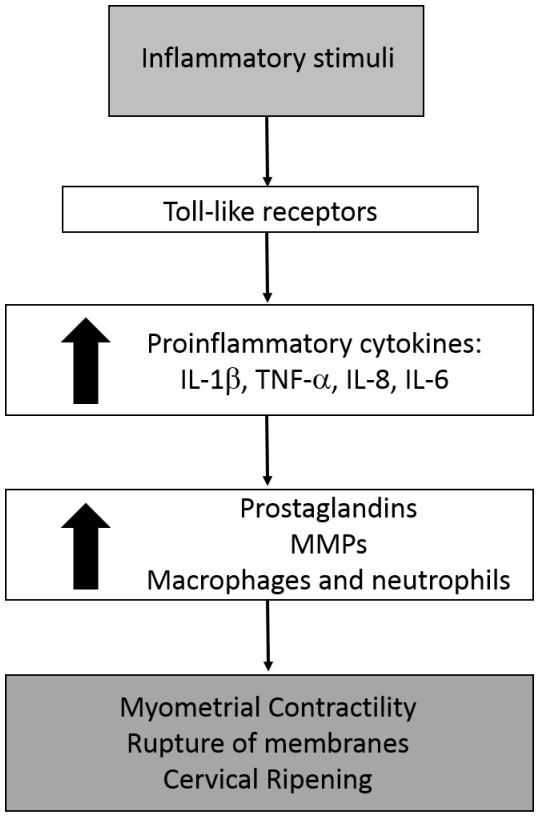

Figure 1.17: **Involvement of cytokines in parturition.** Stimulation of TLRs and secretion of pro-inflammatory cytokines are involved in the onset of the parturition process. Adapted from Challis et al. [2009].

Functions of TNF-α during Pregnancy

The pro-inflammatory cytokine TNF-α has been observed in the syncytiotrophoblast, decidual and epithelial cells in maternal tissue during the first trimester as well as in syncytiotrophoblast, villous stromal cells and decidual cells in term placentas [Chen et al., 1991]. During the first trimester TNF-α inhibits trophoblast migration [Bauer et al., 2004; Otun et al., 2011]. Another role of TNF-α in the placenta is its involvement in trophoblast turnover and renewal [Yui et al., 1994]. Diseases such as spontanous abortion, pre-term labour and pre-eclampsia are associated with a shift to Th1 cytokines including TNF-α [Shaarawy and Nagui, 1997; Sadowsky et al., 2006; Sharma et al., 2007].

1.2.8.2 Fetal Immunotolerance

Several maternal immune cells are present in the pregnant uterus such as uterine natural killer cells (uNKs), macrophages, dendritic cells (DCs) and regulatory T cells (Tregs) [Bulmer et al., 2010]. Since the embryo and fetus are semiallogeneic tissues, it is surprising that no rejection takes place. On the contrary, an active maternal immunological response is observed which establishes a milieu in the uterus in which the fetus is tolerated.

Important in this process of tolerance are several maternal immune cells. Macrophages are the second most abundant group of immune cells in the decidua [Bulmer and Johnson, 1984] and they are involved in phagocytosis of cell debris, immunosuppression and preventing maternal T lymphocyte activation [Abrahams et al., 2004; Nagamatsu and Schust, 2010; Heikkinen et al., 2003]. They also have an immunosuppressive character producing the anti-inflammatory cytokine IL-10 [McIntire et al., 2008]. Furthermore, dendritic cells (DCs) at the interface between mother and fetus are tolerogenic and instead of presenting antigens their function is to expand a regulatory T cells subpopulation (Treg) [Blois et al., 2004, 2007; Amodio et al., 2013; Carosella et al., 2011]. The Tregs ($CD25^+/CD4^+/FoxP3^+$) are extremely important in the maternal tolerance of the fetus and in the maintenance of a successful pregnancy as a decreased number of Tregs has been observed in miscarriages [Heikkinen et al., 2004; Saito et al., 2005; Jin et al., 2009; Winger and Reed, 2011]. Furthermore important for the maternal immunotolerance is that trophoblast cells do not express classical HLA class Ia and class II antigens on their surface like other eukaryotic cells, instead they express HLA-G proteins which contribute to the immunotolerance in several ways [Hunt et al., 2005].

1.3 Stress during Pregnancy

A successful placentation process is very important for the optimal development of the fetus. Stressors can disrupt the placentation processes of placental growth, differentiation and adaptation. If components of the placentation process fail and lead to placental insufficiency, development of the fetus can be impaired, for example placenta-related diseases such as pre-eclampsia (Chapter 1.3.1.1) and intrauterine growth restriction (IUGR) (Chapter 1.3.1.2) can occur. Stress, which can occur in many different forms such as maternal malnutrition, maternal pre-conditions (obesity, diabetes) (Chapter 1.3.2), placental infection as physical stress, but also psychological stress can have adverse effects on the placentation process. Also glucocorticoids are involved in placental pathologies and in the so-called process of fetal programming (Chapter 1.3.3).

1.3.1 Placenta-related Diseases

1.3.1.1 Pre-eclampsia

Pre-eclampsia is a common complication with an incidence of 2-8% of pregnancies [Steegers et al., 2010]. In 75% of the cases, the disease develops at the end of the pregnancy with a mild phenotype. However, pre-eclampsia causes maternal and perinatal morbidity and mortality, including maternal renal or liver failure, stroke, fetal growth restriction, preterm delivery, hypoxic injury, and death [Duley, 2009]. The clinical criteria are new hypertension (diastolic blood pressure of $\geq$ 90 mm Hg) and proteinurea ($\geq$ 300 mg in 24 h) at or after 20 weeks of gestation [Milne et al., 2005]. Pre-eclampsia can develop into the more severe forms of the HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome and eclampsia [Aloizos et al., 2013; Douglas and Redman, 1994].

In pre-term pre-eclampsia the placental weight is decreased, whereas in term pre-eclampsia its weight shows inconsistent and variable changes (U-shaped distribution) [Dahlstrøm et al., 2008]. In severe pre-eclampsia, placentas are characterized by placental poor perfusion and insufficiency which can have detrimental effects on fetal development. Specifically, the invasion of the extravillous trophoblasts (EVTs) and the subsequent remodelling of the maternal spiral arteries is impaired resulting in high resistance vessels with less blood supply [Meekins et al., 1994; Matijevic and Johnston, 1999; Naicker et al., 2003]. Consequences of altered invasion and blood flow are elevated levels of oxidative stress, hypoxia and endothelial dysfunction [Roberts et al., 1991a,b]. Further hallmarks of pre-eclamptic placentas are poor trophoblastic differentiation, endothelial damage, necrosis and hypoxic lesions [Red-

line and Patterson, 1995; Stanek and Weng, 2007]. Also involved in the pathology of pre-eclampsia is an excessive maternal inflammatory response [Redman et al., 1999].

At the molecular level, several abnormalities can be observed in maternal circulation of pre-eclamptic patients and in pre-eclamptic placentas. The expression of factors involved in angiogenesis have been shown to be altered in pre-eclampsia. The proangiogenic placental growth factor (PlGF) is decreased in women with pre-eclampsia, whereas antiangiogenic factors such as sVEGFR-1 (soluble vascular endothelial growth factor receptor-1, synonym is sFlt-1) and sEng (soluble endoglin) are increased [Koga et al., 2003; Levine et al., 2006; Romero et al., 2008; Gu et al., 2008]. With regards to the maternal inflammatory response, an increased activation of NFκB and elevated levels of cytokines such as TNF-α have been observed in women with pre-eclampsia [Luppi et al., 2006; Conrad et al., 1998]. TNF-α is of special interest as it is known to be involved in endothelial activation and dysfunction which is a hallmark of pre-eclampsia [Pober and Cotran, 1990]. Endothelial dysfunction is detected by higher levels of marker molecules of endothelial activation such as thrombomodulin, PAI-1, von Willebrand factor, and fibronectin in pre-eclamptic women [Deng et al., 1994; Shaarawy and Didy, 1996].

In the pre-eclamptic placenta, several ultrastructural changes can be observed. A decreased expression of Syncytin-1 and -2 is shown by several groups [Knerr et al., 2002; Chen et al., 2006; Langbein et al., 2008; Vargas et al., 2011]. Apoptosis is increased in trophoblasts from pre-eclamptic placentas, specifically an increase in activated caspase-3, elevated M30 staining (detects cleaved cytokeratin-18), and a decreased expression of the anti-apoptotic Bcl-2 have been shown in pre-eclamptic placentas [Aban et al., 2004; Austgulen et al., 2004; Ishihara et al., 2002; Longtine et al., 2012]. Furthermore, expression of placental 11β-HSD2 has been shown to be reduced in pre-eclampsia [Causevic and Mohaupt, 2007].

1.3.1.2 Intrauterine Growth Restriction

Intrauterine growth restriction (IUGR) is a complication of pregnancy whereby the fetus does not reach its genetically pre-determined growth potential due to a pathological cause. IUGR is different from small-for-gestational age (SGA) whereby the fetus's weight is less than the 10th percentile for gestational age [Battaglia and Lubchenco, 1967], but no obstetric complications are observed. A better predictor for perinatal morbidity and mortality is a fetal weight less than the 3rd percentile for their gestational age according to McIntire et al. [1999]. The incidence of IUGR

is 3-7% [Romo et al., 2009]. In addition to the increased perinatal morbidity and mortality in IUGR [Bernstein et al., 2000; Gilbert and Danielsen, 2003], the fetus is predisposed for developing certain diseases in its adult life (see chapter 1.3.3).

IUGR can occur due to genetic causes such as aneuploidies or due to environmental causes such as viral infection, drugs including smoking, and placental disorders such as pre-eclampsia [Maulik, 2006; Longo et al., 2014; Mund et al., 2013].

The placenta from IUGR pregnancies is characterized by a decreased weight, a greater thickness, ischemic thrombotic lesions, and excessive tissue injury such as fibrinoid depositions [Teasdale, 1984; Nordenvall et al., 1991; Krebs et al., 1996; Macara et al., 1996]. Furthermore, a decrease in vascularization and in syncytiotrophoblast surface area is observed in IUGR placentas [Chen et al., 2002; Junaid et al., 2014; Teasdale and Jean-Jacques, 1988]. As in pre-eclampsia, a placental malperfusion because of an inadequate spiral arterie remodeling by trophoblasts is observed in IUGR [Lyall et al., 2013]. This leads to the phenomena of hypoxia, re-oxygenation and oxidative stress within the placental tissue [Takagi et al., 2004; Kimura et al., 2013].

At the molecular level, abnormalities in placentas from IUGR pregnancies have been identified. Defects in placental transport function in IUGR have been observed, namely a decreased activity of the System A amino acid transporter and the taurine transporter as well as a decreased placental transport of leucin and lysin which leads to nutrient restriction of the fetus [Norberg et al., 1998; Roos et al., 2004; Jansson et al., 1998]. Potentially because of ER stress placental translation is inhibited and the AKT-mTOR (Protein kinase B-mammalian target of rapamycin) pathway, which is involved in the proliferation and cell growth regulation, is impaired in IUGR placentas [Yung et al., 2008; Roos et al., 2007]. Possibly the insulin-like growth factor (IGF) axis is also involved in the pathogenesis of IUGR as a reduced expression of IGFs has been detected in IUGR placentas [Randhawa, 2008]. Regarding cell turnover, enhanced apoptosis appears to be present in IUGR placentas [Smith et al., 1997a; Ishihara et al., 2002; Crocker et al., 2003]. For example, Levy et al. [2002] detected an elevated expression of the pro-apoptotic molecule p53 in growth-restricted placentas and Endo et al. [2005] observed an increased expression of caspase-3 in IUGR placentas. Also the differentiation process of cytotrophoblasts might be impaired as Ruebner et al. [2010] showed a decreased expression of the fusogenes Syncytin-1 and -2 and of the MFSD2 receptor (receptor for Syncytin-2) in IUGR placentas. Moreover, placental 11β-HSD2 expression has been shown to be decreased in IUGR [Shams et al., 1998; McTernan et al., 2001; Dy et al., 2008; Börzsönyi et al., 2012].

1.3.2 Effects of Maternal Pathologies on Placentation

1.3.2.1 Diabetes

Diabetes during pregnancy can be pre-existing (diabetes mellitus type 1 and 2 (T1DM and T2DM) = pregestational diabetes (PGD)) or can be newly developed as gestational diabetes mellitus (GDM). Diabetes can cause several complications during pregnancy, especially when it is not well treated. Abnormalities in the placenta are observed as well as programming effects on the offspring can occur (Chapter 1.3.3) [Daskalakis et al., 2008; Dabelea et al., 2000; Boney et al., 2005; Yogev and Visser, 2009]. Furthermore, fetal outcomes tend to be characterized by macrosomia (birth weight above the 90th percentile) and excessive fetal fat accumulation and PGD leads to a higher risk of pre-term delivery (depending on the level of glycemic control, prepregnancy BMI, weight gain during pregnancy and the presence of pre-eclampsia) [He et al., 2015; Desoye and van Poppel, 2015; Melamed et al., 2008].

Diabetes leads to a hyperglycemic environment which is different from the maternal and fetal environment during normal healthy pregnancy [Taricco et al., 2009]. Also the pro-inflammatory nature of diabetes might alter placental and fetal development [Radaelli et al., 2003]. In case of PGD, the altered environment is present from the start of the pregnancy, which might already affect early stages of placental development, whereas GDM is developing during pregnancy and therefore might affect placental growth and function to later time points. Both types of diabetes lead to a hyperglycemic-induced hyperinsulinemia in the placental and fetal environment [Desoye and van Poppel, 2015].

The placenta in diabetes is characterized by an increase in weight, an enlargement of the surface, increased cytotrophoblast number, more blood vessels, larger calibre of blood vessels and a thicker basement membrane [Taricco et al., 2003; Jirkovská et al., 2002, 2012; Younes et al., 1996]. The hypervascularization and placental capillary dilatation might be evoked by fetal hypoxia. The oxygen supply from the mother to the fetus is reduced because of the maternal hyperglycemia and an elevated level of HbA_{1c} which has a higher oxygen affinity and this leads to fetal hypoxia [Madazli et al., 2008; Teramo, 2010]. Additionally, the fetus in diabetes has a higher oxygen demand because of the hyperglycemia-induced higher aerobic metabolism which results ultimately in fetal hypoxia. Further, oxidative and nitrative stress is elevated in diabetic placentas [Schönfelder et al., 1996; Coughlan et al., 2004; Lappas et al., 2011].

1.3.2.2 Obesity

The prevalence of obesity has increased over the past years and an association between maternal overweight/obesity and adverse pregnancy outcomes has been observed [Ogden et al., 2006; Yogev and Visser, 2009]. Fetal outcomes of obese mothers show complications such as large-for-gestational-age (LGA) newborns, stillbirths and also programming effects for developing obesity, diabetes and the metabolic syndrome in later life [Baeten et al., 2001; Jensen et al., 2003; Ehrenberg et al., 2004; Cnattingius et al., 1998; Nohr et al., 2005; Salihu et al., 2007; Aune et al., 2014; Boney et al., 2005]. Also the obese mothers are at higher risk for developing hypertension, pre-eclampsia, and gestational diabetes during pregnancy [Galtier-Dereure et al., 2000; Roberts et al., 2011a; Stupin and Arabin, 2014].

Obesity is characterized by a chronic systemic low-grade inflammatory milieu (= increased levels of pro-inflammatory cytokines such IL-6, TNF-α and C-reactive protein in the circulation and infiltration of macrophages into white adipose tissue) [Cancello and Clément, 2006; Clement and Langin, 2007]. Also the placenta is exposed to an environment characterized by increased systemic pro-inflammatory cytokines (IL-6, C-reactive protein) and extensive placental macrophage accumulation with local production of pro-inflammatory cytokines (IL-6, TNF-α, IL-1) in obese mothers [Ramsay et al., 2002; Challier et al., 2008]. The placenta expresses elevated levels of IL-1β, IL-8 and MCP-1 (monocyte chemoattractant protein-1) in maternal obesity [Roberts et al., 2011b].

Alterations in placental phenotype from overweight/obese mothers have not been extensively studied yet. However, some interesting placental alterations on molecular level such as impairment of mitochondrial function leading to increased ROS and decreased ATP levels have been observed in placentae from obese mothers which might compromise placental function [Mele et al., 2014]. Maternal obesity is associated with activation of p38-MAPK and STAT3 signalling pathways in the placenta possible evoked by the increased pro-inflammatory milieu [Aye et al., 2014]. The increased levels of IL-1β in placentae from obese mothers might cause impaired placental insulin signalling [Aye et al., 2013] which could then possibly affect placental hormone production and transport of amino acids [Ren and Braunstein, 1991; Aye et al., 2013]. Moreover, Saben et al. [2014] identified different mRNA expression of genes which are involved in hormone and cytokine activity, lipid metabolism and angiogenesis in the placenta from obese mothers (gene ontology analysis). They further detected activation of the inflammatory NFκB pathway in placentae from

obese mothers [Saben et al., 2014], whereas this NFκB activation in placentae from obese mothers was not confirmed in a study by Aye et al. [2014].

1.3.3 Fetal Programming

Fetal programming is the process of fetal adaptive metabolic changes during fetal development which results in lifelong effects, i.e. these individuals are predisposed to develop metabolic and cardiovascular diseases (for example, obesity, diabetes, hypertension) as well as anxiety-related disorders [Reynolds, 2013b]. Figure 1.18 shows a summary of the fetal programming which demonstrates the central role of glucocorticoids (cortisol in humans) in this process. There are mainly two causes for fetal overexposure to glucocorticoids: activation of the maternal HPA axis by nutritional or other stressful stimuli or a reduced placental 11β-HSD2 activity. Also, administration of synthetic glucocorticoids (by risk of pre-term delivery [Smrcek et al., 2005]) can lead to fetal overexposure to glucocorticoids.

Undernutrition plays an important role in fetal programming demonstrated by studies of the Dutch famine in 1944/1945 [Ravelli et al., 1998; Roseboom et al., 2000, 2001, 2006]. The concept that malnutrition is the cause of fetal programming is known as the Barker Hypothesis [Barker, 1998]. Possibly, this hypothesis is linked with the hypothesis that fetal overexposure to glucocorticoids underlie fetal programming [Edwards et al., 1993] as Gardner et al. [1997] and Langley-Evans [1997] showed that maternal glucocorticoids are involved in the low protein diet-induced programming effects. Also other stressful stimuli including psychological stress are associated with low birth weight and neurodevelopmental alterations in the offspring [Buitelaar et al., 2003; Lipkind et al., 2010; Entringer et al., 2010].

Also, the enzyme 11β-HSD2 plays an important role in the fetal programming process. It has been discovered by Benediktsson et al. [1993] that low birth weight is associated with a reduced activity of 11β-HSD2 in rat. Many further studies in humans and animals confirmed the correlation between birth weight and 11β-HSD2 activity and could demonstrate that a reduced expression of 11β-HSD2 leads to a predisposition for developing various diseases in later life [McTernan et al., 2001; Cottrell and Seckl, 2009].

There is also the possibility of an indirect effect of glucocorticoids on fetal development and programming. Excess glucocorticoids within the placental tissue might alter placental growth and function which then causes or contributes to fetal growth and programming [Braun et al., 2013].

Cortisol is important during fetal development because it is involved in the preparation of the fetus from intra- to extrauterine life, specifically it is involved

in the maturation of lung, liver, kidney and gut [Fowden et al., 1998]. However, excess cortisol levels alter fetal tissue maturation and gene transcription and likely epigenetic modification of genes take place in the programming process [Moisiadis and Matthews, 2014]. Also, during fetal development, excess glucocorticoids have been shown to cause alterations of the fetal HPA axis which persists throughout life and this is involved in the increased susceptibility of the individuals to develop metabolic, cardiovascular and psychiatric disorders in later life [Reynolds, 2013a].

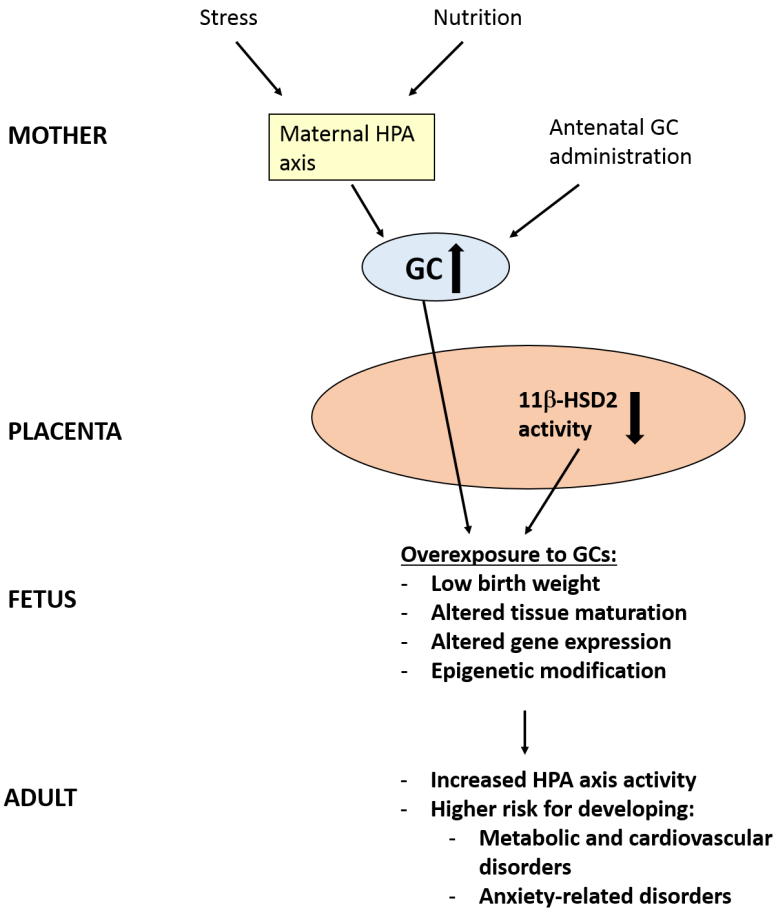

Figure 1.18: **Diagram of Fetal Programming.** Fetal overexposure to glucocorticoids due to higher maternal cortisol levels or to a reduction of placental 11β-HSD2 activity can result in fetal programming which leads to a predisposition of the individuals to develop metabolic, cardiovascular and anxiety-related disorders in later life. Adapted from Reynolds [2013b].

1.4 Thesis Aims

In this thesis, I wanted to investigate trophoblast molecular and cellular responses (placental cell turnover, differentiation, apoptosis, hormone production and expression of molecules involved in the stress response and glucocorticoid action molecular machinery) to hormones and mechanisms controlling placental adaptation processes and stress responses (CRH, glucocorticoids and the enzyme 11β-HSD2).
In detail, aims of this study were:

1. Characterization of the placental explant tissue model to study CRH effects on placenta biology processes such as proliferation, apoptosis and hormone production

2. Elucidation of the BeWo trophoblast differentiation process and effects on cell fusion and endocrine capacity, cell viability and apoptosis, as well as expression of molecules involved in the cellular stress response and local glucocorticoid action such as CRH and its receptors, gluco- and mineralocorticoid receptor and the enzyme 11β-HSD2

3. Characterization of potential novel roles of the placental enzyme 11β-HSD2 in BeWo cell biology besides its role in cortisol inactivation

4. Investigation of BeWo cell glucocorticoid responsiveness

Chapter 2

Material and Methods

2.1 Cell Culture

2.1.1 Placental Explant Culture

Medium for Placental Explant Culture

CMRL-1066 (10X, Gibco, Life Technologies) was supplemented as follows to make 1 L of complete CMRL-1066 medium: 100 mL of 10X CMRL-1066 concentrate was diluted with 800 mL of MilliQ water. 2.2 g $NaHCO_3$ (Sigma-Aldrich), 100 mg streptomycin sulphate (Sigma-Aldrich), 60 mg Penicillin G (1000000 units, Sigma-ALdrich), 100 μg hydrocortisone (Sigma-Aldrich), 1 mg insulin (Sigma-Aldrich), 100 μg retinol acetate (Sigma-Aldrich), 100 mg L-glutamine (Sigma-Aldrich), and 50 mL FBS were added. Medium was filled up with MilliQ water to 1 L, pH was adjusted to 7.4, and medium was sterile filtered. For end concentrations, see table 2.1.

Ingredient	End concentration
Streptomycin Sulphate	100 μg/mL
Penicillin G	100 IU/mL
Hydrocortisone	0.1 μg/mL
Insulin	1 μg/mL
Retinol Acetate	0.1 μg/mL
L-Glutamine	100 μg/mL
FBS	5%

Table 2.1: **End concentrations of supplements added to CMRL-1066 cell culture medium for placental explant culture.**

Placental Explant Culture

Placentae were obtained from healthy individuals by caesarean section after written consent and the project had ethical approval by local LREC. Demographic data of the study subjects were not collected as our study design did not require these data. The design of the study was to conduct different treatments (CRH, LPS) on placental explants from healthy individuals to test whether these different treatments evoke different responses by the explant tissue. This is similar to studies from Audette et al. [2010], Cindrova-Davies et al. [2007], Novembri et al. [2011], Polliotti et al. [1990] and Simán et al. [2001] in which no demographics are presented. (In contrast to this are studies that compare placental explants from healthy patients to placental explants from patients with pregnancy-related pathologies such as pre-eclampsia or IUGR and in these studies demographics of the study subjects are provided [Crocker et al., 2004; Matos et al., 2014; Smith et al., 1998b; Unek et al., 2014].)

Placentae were washed 8-10 times with sterile PBS to remove maternal blood. Chunks were cut out and washed again once in PBS, and twice in complete CMRL-1066 medium. In a Petri dish containing complete CMRL-1066 medium, small fragments (around 3 mm) were cut and 3 of these fragments were cultured in one well of a 12-well Netwell plate (15 mm Netwells, membrane mesh size 74 μm, Corning, Appleton Woods). Wells were filled with 1.5 mL complete CMRL-1066 medium (see above 2.1.1 for details). In all experiments, medium with the appropriate stimuli was replaced every 24 h. Placental explants were cultured for up to 6 days in an incubator (37°C, 5% CO_2, 20% O_2).

For hormone measurement experiments, hormone concentrations were determined (Chapter 2.6 for ELISA details) every 24 h in the cell culture supernatant. On

the last day, placental fragments were washed with PBS, then frozen at -80°C and protein concentration was determined at a later time point. After thawing, explant fragments were centrifuged at maximum speed for 20 s to remove PBS residue. 50 μL 0.3 M sodium hydroxide was added and incubated for 5 min in a 70°C heating block, vortexing several times. The protein concentration was determined using the BCA assay (Chapter 2.4.2 for details) after adding 450 μL H_2O.

For immunohistochemistry (IHC) experiments, on the last day placental explants were washed with PBS and put into formalin (Chapter 2.5.2 for immunohistochemistry protocol). Figure 2.1 shows an example of an IHC of placental explant cultured for five days and stained with haematoxylin.

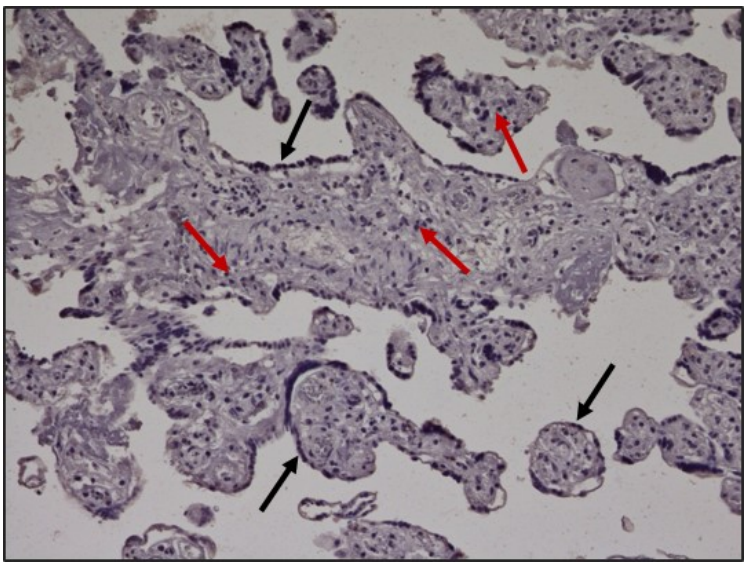

Figure 2.1: **IHC of placental explant.** Haematoxylin staining of placental explant. Blank arrows show the syncytiotrophoblast and red arrows show the cytotrophoblast cells.

For mRNA expression experiments, on the last day placental explants were washed with PBS, then snap frozen at -80°C and mRNA was isolated at a later time point. For mRNA isolation, explants were homogenized using an autoclaved plastic pestle before 500 μL mRNA lysis buffer from GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) was added and the protocol from the kit manufacturer was followed. See Chapter 2.3 for details of mRNA quantification.

For protein expression experiments, on the last day placental explants were washed with PBS, then snap frozen at -80°C and protein lysates were prepared at

a later time point. For lysate preparation, explants were homogenized using an autoclaved plastic pestle and 500 μL RIPA buffer (Santa Cruz) was added before protein concentration was determined using the BCA assay (Thermo Scientific) and Western blot was performed (see Chapter 2.4 for details for protein quantification).

2.1.2 Cell Line Culture

Medium for Cell Line Culture

BeWo cells were cultured in Ham's F12K (Kaighn's) medium (Life Technologies) supplemented with 10% heat-inactivated BenchMark™ FBS (Triple 0.1 μm Sterile-Filtered, Gemini Bio-Products) and 1% Penicillin Streptomycin (5000 U/mL Penicillin, 5000 μg/mL Streptomycin, Life Technologies) (= complete F12K medium).

HeLa and HEK-293 cells were cultured in DMEM high glucose medium (Life Technologies) supplemented with 10% heat-inactivated BenchMark™ FBS (Triple 0.1 μm Sterile-Filtered, Gemini Bio-Products) and 1% Penicillin Streptomycin (5000 U/mL Penicillin, 5000 μg/mL Streptomycin, Life Technologies) (= complete DMEM medium).

BeWo Cell Culture

BeWo cells (ATCC® CCL-98™) were removed from liquid nitrogen, warmed in a water bath for 2-3 min until thawed, diluted with fresh complete F12K medium, transferred in a cell culture flask, and incubated in an incubator (37°C, 5% CO_2, 20% O_2) for 24 h. Then, the medium was changed and the cells were incubated further for 3-4 additional days before splitting.

To split BeWo cells, medium was aspirated and the cells were washed once with PBS before incubating them with 0.05% Trypsin-EDTA (Gibco®, Life Technologies) for 1-2 min in the incubator. Fresh complete F12K medium was added to the trypsinized cells and 1/6 of the cell suspension was transferred in a new cell culture flask. The cells were split every 3-4 days and experiments were performed with cells of less than 30 passages.

For cell freezing, cells were centrifuged after trypsinization for 5 min at 125g. Medium was aspirated, cells were diluted in 5-10% DMSO in complete F12K medium and placed in a cryofreezing container (Fisher Scientific) in -80°C for 24 h before storing them in liquid nitrogen.

HEK-293 and HeLa Cell Culture

HEK-293 (ATCC® CRL-1573™) and HeLa (ATCC® CCL-2™) cells were taken out of liquid nitrogen, warmed in a water bath for 2-3 min until thawed, diluted with 10 mL fresh complete DMEM, and centrifuged for 5 min at 125g. Supernatant was aspirated, cell pellet was resuspended in fresh complete DMEM, cells were transferred in a new cell culture flask, and incubated in an incubator (37°C, 5% CO_2, 20% O_2) for 3-4 days before splitting.

To split the HEK-293 and HeLa cells, medium was aspirated and the cells were washed once with PBS before incubating them with 0.05% Trypsin-EDTA (Gibco®, Life Technologies) for 1 min at room temperature (HEK-293) or 2 min in the incubator (HeLa). Ten mL of fresh complete DMEM medium was added to the trypsinized cells and they were centrifuged for 5 min at 125g. The supernatant was aspirated, the cell pellet was resuspended in fresh complete DMEM, 1/6 of the cell suspension was transferred in a new cell culture flask, and incubated in a CO_2 incubator (37°C, 5% CO_2, 20% O_2). The cells were split every 3-4 days and experiments were performed with cells of less than 30 passages.

For cell freezing, cells were centrifuged after trypsinization for 5 min at 125g. Medium was aspirated, cells were diluted in 5-10% DMSO in complete DMEM medium and placed in a cryofreezing container (Fisher Scientific) in -80°C for 24 h before storing them in liquid nitrogen.

2.2 Treatments

2.2.1 Preparation of Stock Solutions and End Concentrations

CRH Treatment

CRH (Bachem or Calbiochem) was dissolved in 10% acetic acid to make a stock solution of 1 mM. BeWo cells and placental explants were treated with 0.1 or 1 μM CRH for various time points depending on the experimental protocol. These concentrations were chosen because of successful experiments performed with these concentrations by members of our group.

LPS Treatment

LPS (from *Escherichia coli*, Sigma-Aldrich) was dissolved in CMRL-1066 medium to make a stock solution of 2 mg/mL. Placental explants were treated with 10 μg/mL

for 4 to 6 days depending on the experimental protocol (medium was changed every 24 h). This concentration was chosen because of successful experiments by Okada et al. [1997], Uh et al. [2008], Torricelli et al. [2011] and by members of our group.

Forskolin Treatment

Forskolin (from *Coleus forskohlii*, EMD Millipore) was dissolved in DMSO to make a stock solution of 100 mM. BeWo cells were treated with 100 μM forskolin at a confluency between 60 and 80% for 24 or 48 h, depending on the experimental protocol. This concentration was chosen because of successful experiments by Wice et al. [1990], Delidaki et al. [2011] and by members of our group.

Dexamethasone Treatment

Dexamethasone (Sigma-Aldrich) was diluted in 100% ethanol to make a stock solution of 10^{-2} M. BeWo, HEK 293 and HeLa cells were treated with 1 μM dexamethasone for various time points depending on the experimental protocol. This concentration was chosen because of successful experiments by Mark and Waddell [2006] and by members of the NICHD-based group.

Hydrocortisone (= Cortisol) Treatment

Hydrocortisone solution (50 μM, sterile-filtered, BioXtra, suitable for cell culture, Sigma-Aldrich) was used at various concentrations (100 nM - 2 μM) in BeWo cell culture depending on the experimental protocol.

Kinase Inhibitor Treatments

MAPK (p38 and $ERK_{1/2}$) inhibitors SB202190 (In solution (DMSO), Calbiochem), UO126 (stock concentration was 10 mM in DMSO, Calbiochem) as well as PI3K inhibitor Wortmannin (In solution (DMSO), Calbiochem) were used. BeWo cells were pre-treated with the inhibitors SB202190, UO126 and Wortmannin for 24 h at concentrations of 2.5 μM, 10 μM and 300 nM, respectively. BeWo cells were subsequently treated depending on the experimental protocol in the presence of the inhibitors. These concentrations were chosen based on the instructions in the chemical manuals provided by the companies and because of successful experiments by

members of our group.

Staurosporine Treatment

Staurosporine (from *Streptomyces sp*, Sigma-Aldrich) was dissolved in DMSO to make a stock solution of 2 mM. Placental explants were treated with 10 μM staurosporine to induce apoptosis (= positive control for M30-staining and MSD Multiplex ELISA). BeWo cells were treated with 1 μM staurosporine (= positive control for ApoONE® Homogeneous Caspase-3/7 Assay). These concentrations were chosen based on the information provided by the manufacturer's website and because of successful experiments by Jacobsen et al. [1996].

2.2.2 Transfection

2.2.2.1 siRNA Transfection

The siRNA (Control-siRNA A, 11β-HSD2 siRNA from Santa Cruz) was purchased as lyophilized powder and dissolved by adding the appropriate amount of RNase-free water. The dissolved chemicals were vortexed, aliquoted and stored at -20°C.
To transfect BeWo cells with siRNA, cells were seeded in 24- or 6-well plates and grown until they reached a confluency of 50-60%. On the day of transfection, siRNA was diluted in Opti-Mem, transfection reagent (Attractene, Qiagen) was added and the tube was vortexed briefly. During an incubation time of 10-15 min at room temperature, the medium of the cells was aspirated and replaced with Opti-Mem (Life Technologies) containing 5% FBS (Triple 0.1 μm Sterile-Filtered, Gemini Bio-Products). The mixture of siRNA and transfection reagent was added to the cells and the culture plate was rocked gently (see table 2.2 for volumes and concentrations).

well-plate	Volume 5% FBS in Opti-MEM in mL	Volume Opti-MEM in μL	siRNA nM	Attractene in μL
24	0.5	50	15	1.5
6	1.5	100	15	4.5

Table 2.2: **siRNA Transfection.** Volumes and concentrations of siRNA and transfection reagent were chosen based on the manufacturer's instructions and group member's expertise.

2.2.2.2 DNA-Vector Transfection

DNA-Vectors

The pMMTV-luc DNA-vector, which expresses the *firefly*-luciferase under the control of a mouse mammary tumor virus (MMTV) promoter with four glucocorticoid response elements (GREs) (Figure 2.2, Guido et al. [1996]), was a gift from G.L. Hager (National Cancer Institute, MD, USA). The pRShGRα DNA-vector, which expresses the human GR receptor, contains an RSV (Rous sarcoma virus) promoter (Giguère et al. [1986]) and was a gift from Dr. R. M. Evans (Salk Institute, La Jolla, CA, USA). The pGL4.73-Rluc DNA-vector, which expresses the *renilla*-luciferase, contains a Simian virus 40 promoter and was purchased from Promega. The pGL4.73-Rluc vector was used as an expression control vector in reporter-gene-assays.

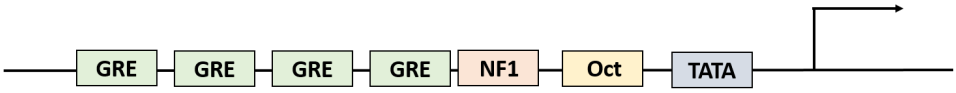

Figure 2.2: **Promoter of the MMTV-luc DNA vector.** Adapted from Guido et al. [1996].

DNA-Vector Transfection Protocol

To transfect BeWo, HEK 293, or HeLa cells with DNA-vectors, cells were seeded in 12- or 6-well plates. On the day of transfection, DNA-vectors and Lipofectamine 2000 (Invitrogen, Life Technologies) were diluted in Opti-MEM (Life Technologies)

seperately before combining them with gentle mixing. During an incubation time of 5 min, medium of the cells was aspirated and replaced with fresh complete medium. The mixture of DNA-vector and transfection reagent was added to the cells and the culture plate was rocked gently (see table 2.3 and 2.4 for volumes and concentrations).

For reporter-gene-assays, pMMTV-luc was co-transfected with pGL.4.73-Rluc with and without pRShGRα in a 12-well plate. After 4 h of transfection, medium was changed and cells were treated with dexamethasone with and without forskolin for 4 or 20 h before performing the Dual-Luciferase-Assay (see subchapter below).

For qRT-PCR experiments, pRShGRα was transfected in a 6-well plate. 4 h after transfection, medium was changed and cells were treated further depending on the experimental protocol with dexamethasone with and without forskolin for 20 h before extracting mRNA (Chapter 2.3).

well-plate	**Volume Opti-MEM in μL**	**Volume medium in mL**	**Volume Lipofectamine in μL**
12	2 x 50	0.5	1.75
6	2 x 150	1	7.5

Table 2.3: **DNA Transfection (Volumes).** Volumes of Opti-MEM, medium, and lipofectamine for DNA-transfection experiments were chosen based on the manufacturer's instructions and group member's expertise.

well-plate	**pMMTV in μg**	**Rluc in μg**	**pRShGRα in μg**
12	0.25	0.025	$\pm$0.2
6			2

Table 2.4: **DNA Transfection (Concentrations).** Concentrations of DNA-vectors for DNA-transfection experiments were chosen based on the manufacturer's instructions and group member's expertise.

Dual-Luciferase Assay

Measurement of luminescence from luciferase-containing DNA-vectors was performed using the Dual-Luciferase® Reporter Assay System (Promega).
After transfection with *firefly*- and *renilla*-containing DNA-vectors and subsequent incubation with glucocorticoids with and without further treatments, cells were once washed with PBS and lysed with 250 μL (for one well in 12-well plate) 1X Passive Lysis Buffer (component of the kit) for 25 min, shaken. 20 μL of supernatant was transferred in one well of a black or white 96-well plate for measurement of luminescence (in relative light units = RLU). Readings were performed with a luminometer.

2.2.3 Treatment Protocols

Figure 2.3 shows the different treatment protocols of cell culture experiments.

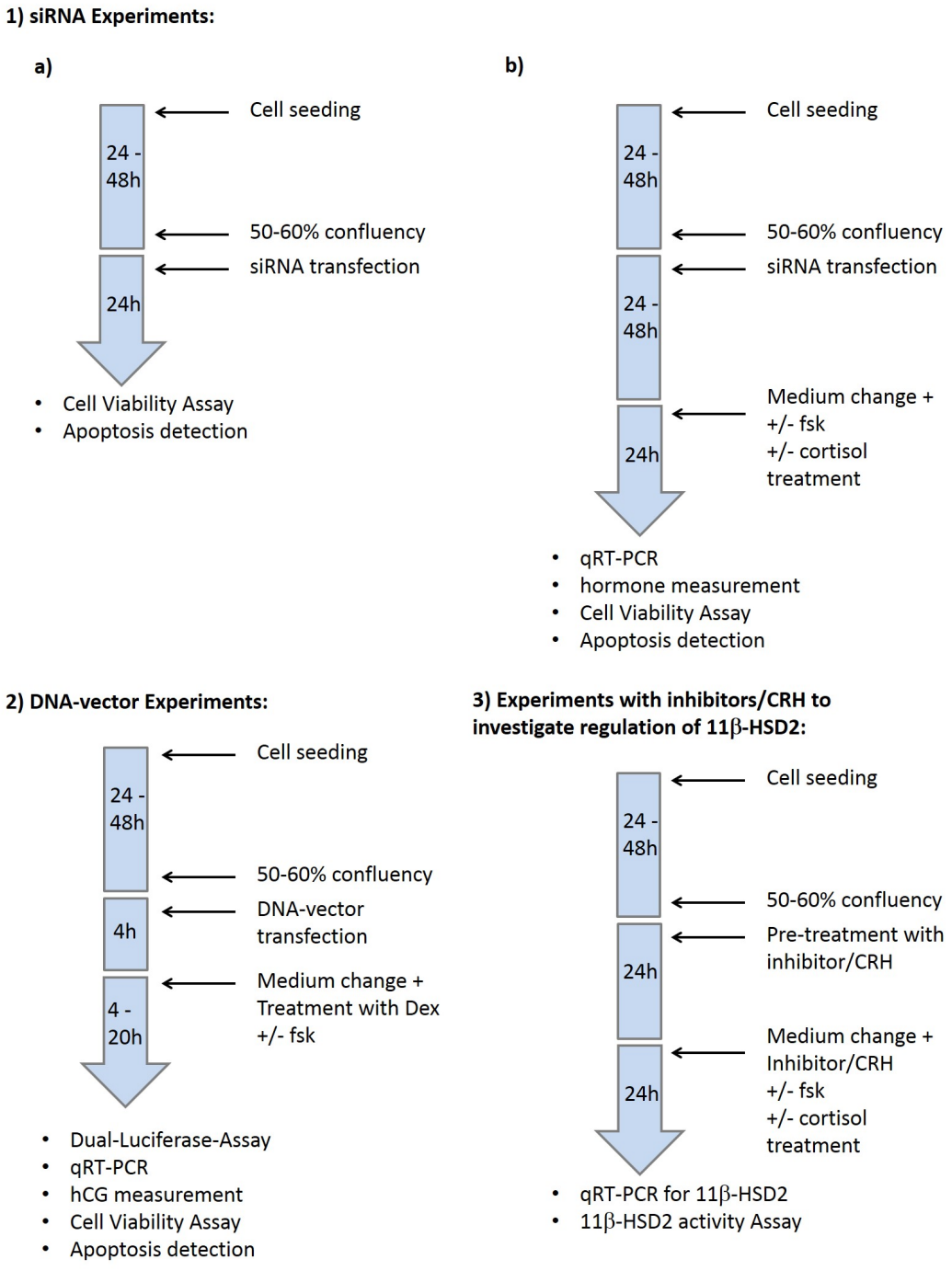

Figure 2.3: **Treatment protocols.** Experimental procedure of **(1)** siRNA transfection in BeWo cells, **(2)** DNA-vector transfection in BeWo, HEK 293 and HeLa cells and **(3)** experiments with inhibitors/CRH to investigate regulation of 11β-HSD2 in BeWo cells. All protocols start with a 24 to 48 h incubation to allow cells to reach cell confluency of 50-60%. Then different treatments were carried out according to the purpose of the experiments.

2.3 mRNA Quantification

2.3.1 mRNA Isolation

Total RNA was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma) according to the manufacturer's instructions. Briefly, 300 μL of lysis buffer (complemented with 1% 2-mercaptoethanol) per well (6-well plate) were used to lyse cells (BeWo, HEK293, HeLa). After transferring the lysate to a filtration column which separates the RNA-containing liquid from cell debris, an equal volume of 70% ethanol was added to the filtered lysate and vortexed. The lysate/ethanol mixture was loaded onto a binding column. Several wash steps with Wash buffer 1 and 2 were performed. 30-50 μL of elution buffer were used to elute the mRNA from the column.

Determination of mRNA (Nanodrop)

The isolated mRNA was quantified by measuring the absorbance at 260 nm using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA quality was verified by assessing the A260/280 and the A260/230 ratio (with the nanodrop) and by performing agarose gel electrophoresis with 1 μg of mRNA (Figure 2.4). Intact total RNA is represented by two bands (28S and 18S rRNA) and the ratio of their intensities should be 2:1 (28S rRNA : 18S rRNA).

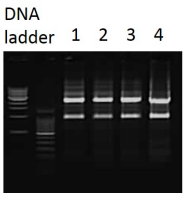

Figure 2.4: **mRNA gel image.** First DNA ladder: DNA marker high molecular weight, second DNA ladder: DNA marker low molecular weight, lane 1-4: 1 μg of isolated mRNA from BeWo cells (lane 1, 2) and from HEK 293 (lane 3, 4).

2.3.2 cDNA Synthesis of isolated mRNA

Isolated mRNA (400 ng) was reverse transcribed using Taqman® Reverse Transcription Reagents (Invitrogen™, Life Technologies). Four hundred ng (in 7.2 μL

of H_2O) was incubated with 12.8 μL mix (Table 2.5) for 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C.

Or 500 ng isolated mRNA was reverse transcribed using reagents from Fermentas in a total volume of 20 μL. Five hundred ng was incubated for 5 min with 1 μL 0.5 μg/μL random hexamers in a volume of 12 μL H_2O at 70°C before adding 8 μL mix (Table 2.6) and incubating for 1 h at 37°C followed by 10 min at 70°C.

Reagent	**Volume in μL**
RT Buffer 10x	2
25 mM Mg Cl_2	4.4
dNTP 2.5 mM	4
Hexamer 50 μM	1
Oligo dT 50 μM	0.5
RNase Inhibitor (20 U/μL)	0.4
MultiScribe™ RT (50 U/μL)	0.5

Table 2.5: **cDNA synthesis of mRNA using Taqman® Reverse Transcription Reagents.** Volumes of reagents needed for cDNA synthesis.

Reagent	**Volume in μL**
5x Reaction Buffer	4
10 mM dNTP mix	2
Ribolock RNase Inhibitor (20 U/μL)	1
Revertaid H-Minus-MuLV RT (2000 U/μL)	1

Table 2.6: **cDNA synthesis of mRNA using the Fermentas Reagents.** Volumes of reagents needed for cDNA synthesis.

2.3.3 Quantitative RT-PCR

2.3.3.1 Taqman®-based quantitative RT-PCR

Messenger RNA expression of 11β-HSD2, Anxa1, Dusp1, Sync1, Sync2, SGK1, ATP1A1, GR, and MR (Table 2.7) were quantified using the Taqman-based quantitative RT-PCR. 18S rRNA was used as a housekeeping gene. 10 μL of cDNA dilution was mixed with 11 μL of mastermix (10 μL 2x Taqman® Gene Expression

Master Mix (Applied Biosystems®, Life Technologies) + 1 μL 10x primer) and incubated using the following program: 2 min at 50°C, 10 min at 95°C, 40x: 15 s 95°C + 1 min 60°C.

Primer	**Catalog number**
11β-HSD2 (HSD11B2)	Hs00388669_m1
Sync-1 (ERVW-1)	Hs00205893_m1
Sync-2 (ERVFRD-1)	Hs01652148_m1
GRα (NR3C1)	Hs00353740_m1
MR (NR3C2)	Hs01031809_m1
Dusp1	Hs00610256_g1
Anxa1	Hs00167549_m1
SGK1	Hs00985033_g1
ATP1A1	Hs00167556_m1
18S rRNA	4319413E
β-actin (ACTB)	4333762T
GAPDH	4333764T

Table 2.7: **Primer for Taqman® qPCR.** Primer were purchased from Life Technologies.

2.3.3.2 SYBR® Green-based quantitative RT-PCR

Primer Design

Primer for SYBR® Green-based quantitative RT-PCR were designed using the programme Primer 3 Plus with the following settings: amplicon size = 70-150 bp, primer size = 18-24 bp, T_m = 55-65°C, and GC content = 40-60%. The target sequence was gained from the PubMed nucleotide or Ensembl database and the primer specificity was verified by making a BLAST or a BLAT search.

Purification of Primer

Lyophilized powder of primer were resuspended in 300 μL distilled water and centrifuged for 5 min at maximum speed to remove debris. Supernatant was transferred to a new tube and 6 μL of 5 M NaCl and 600 μL of 100% EtOH were added. After

vortexing briefly, the samples were stored at -20°C for 30 min and then centrifuged for 30 min at 4°C at maximum speed. Supernatant was aspirated, 500 μL of 70% EtOH added and centrifuged for 5 min at maximum speed. Supernatant was aspirated and pellet was dried. Dry pellet was resuspended in 200 μL of TE and concentration was measured with the nanodrop. Molarity of the primer solution was calculated with the following equation:
DNA (pmol/μL = μM) = DNA concentration (ng/μL) x 1000 / Molecular Weight (MW)
MW = 330 x number of nucleotides
A stock solution of 10 μM was stored at -20°C.

SYBR® Green-based quantitative RT-PCR of mRNA

Messenger RNA expression of Per1, Gilz, CRH, CRH-R1, CRH-R2, ASCT2 and MFSD2 (Table 2.8) were quantified using the SYBR® Green-based quantitative PCR. As a housekeeping gene GAPDH, β-actin and RPLP0 mRNA was used. Five μL of cDNA dilution was mixed with 15 μL of mastermix (10 μL 2x *Power* SYBR® Green PCR Master Mix (Applied Biosystems®, Life Technologies) + 1 μL 10 μM primer F + 1 μL 10 μM primer R + 3 μL H_2O) and incubated using the following program: 10 min at 95°C, 40x: 15 s 95°C + 1 min 60°C. In order to confirm a single PCR product, melting curve analysis has been performed after the amplification of the PCR product (Figure 2.5).

Primer	**Sequence**
GAPDH F	5'-GAGTCAACGGATTTGGTCGT-3'
GAPDH R	5'-GACAAGCTTCCCGTTCTCAG-3'
β-actin (ACTB) F	5'-CGGCATCGTCACCAACTG-3'
β-actin (ACTB) R	5'-AAGGTGTGGTGCCAGATTTTCT-3'
RPLP0 F	5'-CAATGGCAGCATCTACAACC-3'
RPLP0 R	5'-TGATGCAACAGTTGGGTAGC-3'
Per1 F	5'-CACTGGCCTGTGTCAAGC-3'
Per1 R	5'-GTGTACTCAGACGTGATGTG-3'
GILZ F	5'-GATGTGGTTTCCGTTAAGC-3'
GILZ R	5'-CTCTCTCACAGCATACATCAG-3'
CRH F	5'-TCCCATCTCCCTGGATCTCAC-3'
CRH R	5'-GTGAGCTTGCTGTGCTAACTGCT-3'
CRH-R1 F	5'-CGCATCCTCATGACCAAGCT-3'
CRH-R1 R	5'-TCACAGCCTTCCTGTACTGAATG-3'
CRH-R2 F	5'-TGCGGAGCATTCGCTGT-3'
CRH-R2 R	5'-TTTCGCAGGATAAAGGTGGTG-3'
ASCT2 F	5'-ACATCCTGGGCTTGGTAGTG-3'
ASCT2 R	5'-GGGCAAAGAGTAAACCCACA-3'
MFSD2 F	5'-CCTTGTTTCCAGGACCTCAA-3'
MFSD2 R	5'-GAAGTAGGCGATTGGCTCAG-3'

Table 2.8: **Primer for SYBR® Green qRT-PCR.**

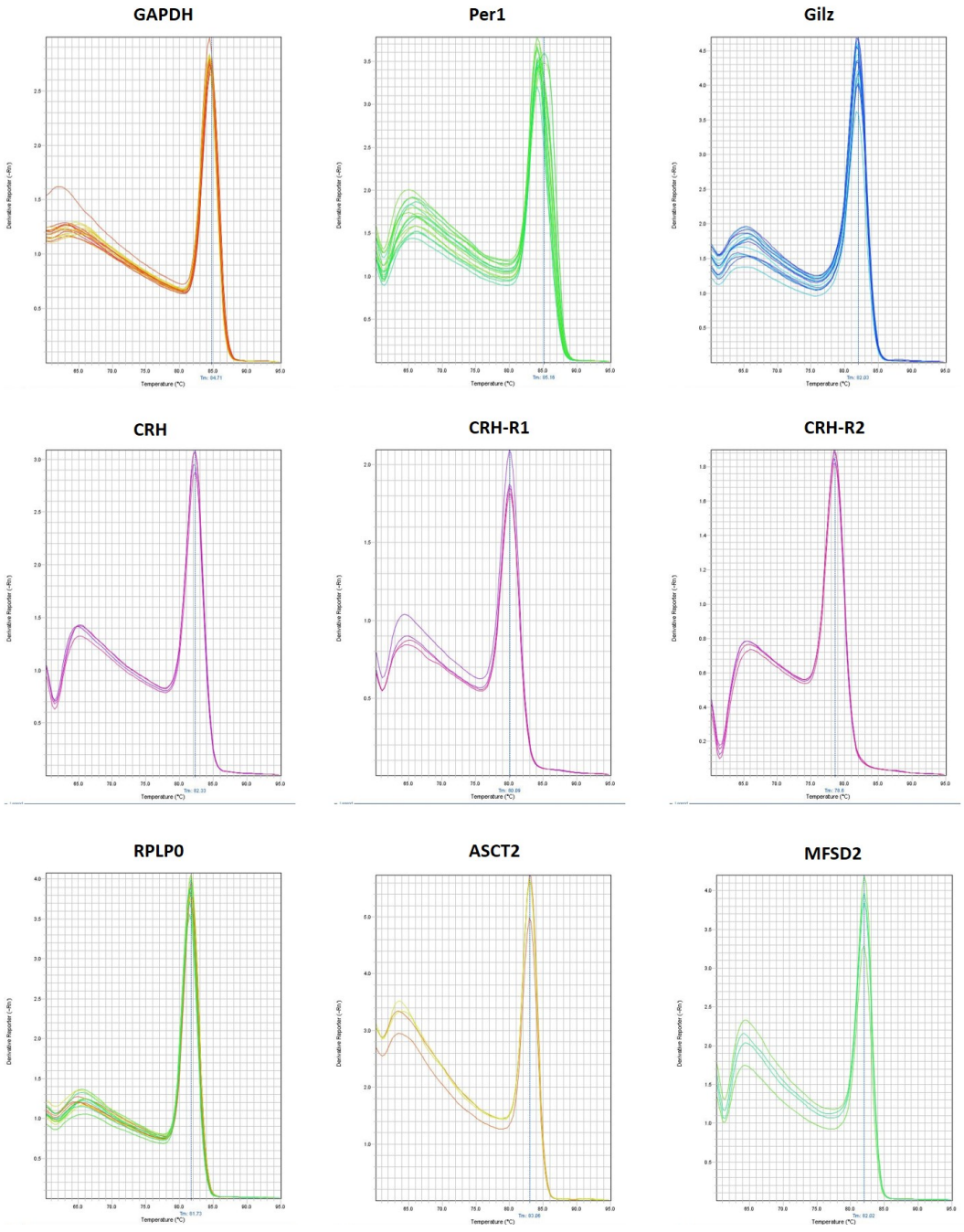

Figure 2.5: **Melting Curves of SYBR Green qRT-PCR Products.** Melting Curves of products of GAPDH, Per1, Gilz, CRH, CRH-R1, CRH-R2, RPLP0, ASCT2, and MFSD2 are shown, representative graphs are shown.

2.3.3.3 Analysis of qRT-PCR

The results were analysed with the ΔΔCt method to calculate the fold mRNA expression compared to the basal treatment condition. The following equations were used to calculate the relative quantity (RQ):

$\Delta Ct = Ct_{(\text{gene of interest})} - Ct_{(\text{housekeeping gene})}$

$\Delta\Delta Ct = \Delta Ct_{(\text{sample})} - \Delta Ct_{(\text{control})}$

$RQ = 2^{-\Delta\Delta Ct}$

2.4 Protein Quantification

2.4.1 Preparation of Protein Lysate

At the end of the experiment, medium was aspirated and the cells were washed with ice-cold PBS before adding RIPA buffer (200 μL in one well of a 6-well plate or 50 μL in one well of a 24-well plate) supplemented with 10 μL PMSF, 10 μL orthovanadate and 10 μL protease inhibitor cocktail per mL RIPA buffer (components of the kit). Protein lysate was transferred in a tube and centrifuged for 10 min (4°C, maximal speed). The supernatant was transferred in a new tube and stored at -80°C.

2.4.2 Measurement of Protein Concentration

Protein concentration of the protein lysates was measured in duplicates using the BCA Protein Assay Kit (Thermo Scientific) according to manufacturer's instructions. Briefly, protein lysate was diluted 1:5 in RIPA buffer, and 25 μL of each sample was used for the assay. Standard curve samples were diluted in RIPA buffer according to the manufacturer's dilution protocol to generate samples with a concentration between 25 and -2000 μg/mL BSA. Two hundred μL of working reagent (component provided by the kit) was added to each well and the microtiter plate was incubated at 37°C for 15 min before measuring the absorbance at 590 nm. With the help of the linear equation for the standard curve (Figure 2.6), the protein concentration for each sample was calculated.

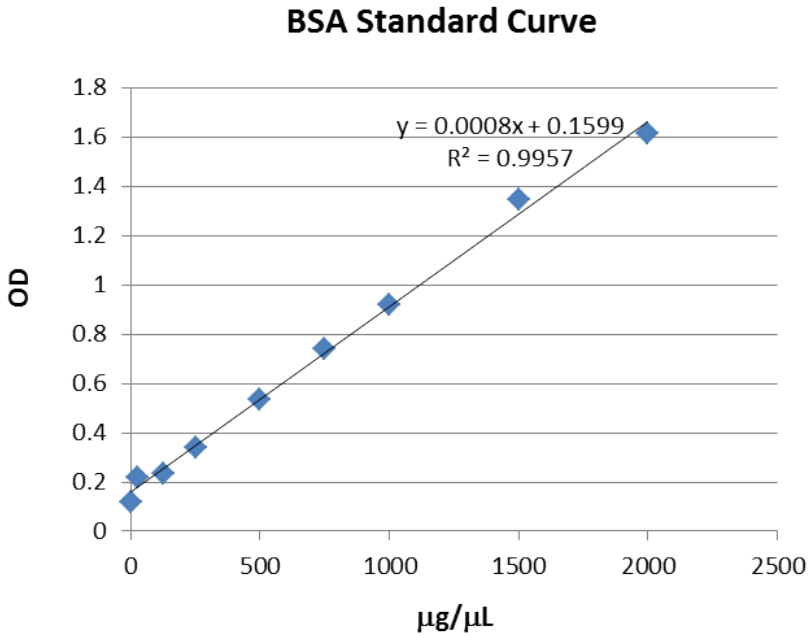

Figure 2.6: **BSA standard curve.** BSA standard (from BCA Protein Assay Kit) was diluted in RIPA buffer to produce a standard curve with concentrations between 25-2000 μg/mL.

2.4.3 Laemmli Lysate

Protein samples were appropriately diluted in Laemmli 2x Sample Buffer (Sigma-Aldrich) to achieve an end concentration of 0.5 μg/μL and incubated at 95°C in a heating block for 5 min before cooling the samples on ice and storing them at -20°C.

2.4.4 Gel Electrophoresis

Gel Preparation

The resolving gel (Table 2.9) was poured between 2 glass plates of the Mini-Protean III Electrophoresis Cell and was overlaid with distilled water to polymerize within 30 min. The water was removed with Watmann filter paper, the stacking gel (Table 2.9) was poured on top of the resolving gel, a comb was inserted and the gel polymerized within 20 min.

Solution components (for 8 gels)	**Resolving Gel**	**Stacking Gel**
Distilled H_2O	9.9 mL	5.8 mL
Tris HCl 1.5 mM pH 8.8	6.25 mL	-
Tris HCl 0.5 mM pH 6.8	-	2.5 mL
Protogel	8.35 mL	1.5 mL
10% SDS	250 μL	100 μL
APS	250 μL	100 μL
TEMED	25 μL	15 μL

Table 2.9: **Volumes for gel preparation.** Resolving and stacking gel were prepared according to the volumes of this table. Protogel is an acrylamide/methylene bisacrylamide (37.5/1 ratio) solution (National Diagnostics). The ammonium persulphate (APS) was freshly prepared as a 10% solution with distilled water.

Electrophoresis

Fifteen μL of Laemmli lysate was loaded in one slot of a 4-20% Tris-Glycin gel (Novex 4-20% Tris-Glycin Express Kit 1.0 mm 12 well, Life Technologies) or self-made gel (see above), as a marker 10 μL of PageRuler Prestained Protein Ladder (Thermo Scientific) was used. Electrophoresis conditions were set to 200 V for 1.5

h. Running Buffer was 10x Tris/Glycin/SDS Buffer (National Diagnostics) or a self-made 10x Running Buffer (30 g Tris-HCl, 144 g glycine, 10 g SDS, distilled water to 1000 mL) diluted with deionized water to obtain a 1x solution.

2.4.5 Blotting

Protein was transferred onto a nitrocellulose membrane (Novex, Life Technologies) for 1 h at 100 V. Transfer buffer was 25x Novex Tris-Glycin Transfer Buffer (Invitrogen, Life Technologies) or a self-made 10x Transfer Buffer (7.575 g Tris-HCl, 36 g glycine, 500 mL methanol, distilled water to 2500 mL) diluted with deionized water and methanol to obtain a 1x solution with 20% methanol.

2.4.6 Visualization of Proteins

2.4.6.1 ECL Detection

Nitrocellulose membranes were blocked for 1 h at room temperature, shaken, with 5% BSA (Sigma-Aldrich) in Tris-buffered saline containing 0.05% Tween 20 (=TBST, 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20). After one wash step with TBST, the membranes were incubated with primary antibody (Table 2.10) diluted in 1% BSA (Sigma-Aldrich) or milk in TBST overnight at 4°C, shaken. The next day, membranes were washed three times with TBST before incubating with secondary antibody (goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP (Santa Cruz)) diluted 1:5000 in 1% BSA or milk in TBST for 1 h at room temperature, shaken. After three wash steps, protein bands were visualized on chemiluminescence film (High performance chemiluminescence film, GE Healthcare) using ECL Western Blotting Detection Reagents (GE Healthcare) and band intensities were quantified using Image J software. Bands of interest were normalized to β-actin or α-tubulin protein expression (Table 2.10). To re-probe the membranes with the primary antibody β-actin or α-tubulin, the membranes were incubated in Restore PLUS Western Blot Stripping Buffer (Thermo Scientific) for 15 min at room temperature, shaken, washed once with TBST and blocked again with 5% BSA or milk in TBST before incubating in primary and secondary antibody as described above.

Primary Antibody	Blocking	Dilution
11β-HSD2 antibody (H-145) (Santa Cruz (sc-20176))	Odyssey Blocking or 5% BSA	1:800 (Odyssey) 1:1000 (ECL)
GR antibody (P-20) (Santa Cruz (sc-1002))	5% BSA or milk	1:200 - 1:500 (ECL)
GR antibody (H-300) (Santa Cruz (sc-8992))	5% BSA or milk	1:200 - 1:500 (ECL)
MR antibody (H-300) (Santa Cruz (sc-11412))	5% BSA or milk	1:200 (ECL)
MR antibody (Abcam (ab-97834))	5% BSA or milk	1:1000 (ECL)
FKBP51 (D-4) (Santa Cruz (sc-271547))	Odyssey Blocking	1:100 (Odyssey)
β-actin (Abcam (ab8226))	Odyssey Blocking or 5% BSA	1:10000 (Odyssey) 1:1000 (ECL)
α-tubulin (Abcam (ab80779))	5% BSA	1:10000 (ECL)

Table 2.10: **Primary antibodies with blocking conditions and dilutions for western blot.**

2.4.6.2 Odyssey Infrared Imaging System

Nitrocellulose membranes were blocked for 1 h at room temperature, shaken, with Odyssey Blocking Buffer (LI-COR Biosciences). The membranes were incubated with primary antibody (Table 2.10) diluted in Odyssey Blocking Buffer overnight at 4°C, shaken. Membranes were washed three times with TBST (24.2 g Tris-base, 80 g NaCl, distilled water to 1000 mL, pH 7.6, 0.1% Tween-20 (= 10x) diluted with distilled water to 1x solution) before incubating with secondary antibody (IRDye 800CW conjugated goat anti-rabbit IgG (H+L) antibody (LI-COR Biosciences) or ALEXA Fluor 680 conjugated goat anti-mouse IgG (H+L) antibody (Life Technologies)) diluted 1:5000 in Odyssey Blocking Buffer for 1 h at room temperature (in the dark), shaken. After three wash steps in TBST and one wash in TBS, protein bands were visualized with the Odyssey Infrared Imaging System and bands were quantified with the Odyssey software version 2.1. To re-probe the membranes with the primary antibody β-actin, the membranes were incubated twice in self-made stripping buffer (25 mM glycine pH 2, 2% (w/v) SDS) for 15 min at room temperature, shaken, washed twice with TBST and blocked again with the Odyssey Blocking Buffer before incubating in primary and secondary antibody as described above.

2.4.6.3 Western Blot Detection of GR and MR

The quantification of GR and MR protein expression was not possible due to problems with antibodies employed to identify GR and MR (Table 2.10, Figure 2.7). Regarding GR, the first GR antibody (sc-1002) did not give a band at the predicted size of 90/95 kDa. The second GR antibody tested (sc-8992) gave white bands on a dark background. This phenomenon is caused by excess of primary and/or secondary antibody and it could have been improved with diluting the primary or the secondary antibody or both. But nonetheless, no pale band was observed at the correct size. Regarding MR, a lot of unspecific bands were observable of which a band above the 100 kDa marker band could potentially be MR as its size is 107 kDA. But due to the various stronger unspecific bands, this band was not regarded as being specific for MR. Despite numerous attempts to improve results with these antibodies, this was not possible.

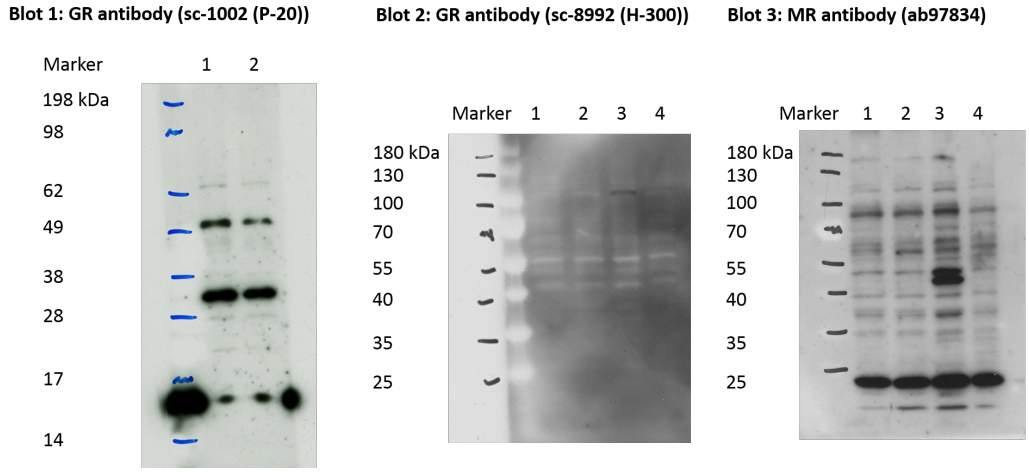

Figure 2.7: **Protein expression of GR and MR in BeWo, HEK 293 and HeLa cells.** Blot 1: Samples were protein lysates from BeWo control (= lane 1) and forskolin-treated BeWo (= lane 2), Blot 2 and 3: Samples were protein lysates from BeWo control (= lane 1) and forskolin-treated BeWo (= lane 2), from HEK 293 control (= lane 3) and HeLa control (= lane 4). Size of GR: 90/95 kDa, size of MR: 107 kDa.

2.5 Microscopy

2.5.1 Immunofluorescence

Immunostaining for fluorescent Microscope

To perform fluorescent immunocytochemistry staining on BeWo cells, the cells were seeded onto sterile glass plates in 6-well plates. After the appropriate treatments, the cells were washed with PBS before fixing them with 4% paraformaldehyd solution for 15 min. After three washing steps with PBS for 5 min, the cells were blocked and permeabilized with 3% BSA in 0.015% Triton-X-100 in PBS for 1 h at room temperature. Then, the cells were washed 3 times with 0.015% Triton-X-100-PBS for 5 min and the primary antibody (1:50 dilutions for 11β-HSD2, E-Cadherin, Syncytin-1, CRH-R1, CRH-R2 primary antibodies), which was diluted in 0.015% Triton-X-100-PBS, was incubated overnight in a wet chamber. The next day, after 3 washing steps with 0.015% Triton-X-100-PBS, the fluorescent secondary antibody (donkey anti-mouse/rabbit/goat ALEXA Fluor 488 or 633 (Life Technologies), 1: 400 diluted in 0.015% Triton-X-100-PBS) was incubated for 1 h at room temperature in the dark. After another 3 washing steps with 0.015% Triton-X-100-PBS and a

final washing step with filtered PBS, the cells were mounted in vectashield mounting medium containing DAPI (Vector laboratories) and the glass slide was placed up-side-down onto a microscope slide and sealed with nail polish. Images were taken using a confocal microscope (SP2, Leica, Milton Keynes, UK).

Vybrant™ Cell-Labeling

BeWo cells were trypsinized and dissolved in serum-free medium at a concentration of 1 x 10^6 /mL. Cells were separated into two populations and either 5 μL of Vybrant DiI or 5 μL of Vybrant DiO cell-labeling solution were added to 1 mL of cell suspension and incubated at 37°C for 20 min. Cells were centrifuged at 1500 rpm for 5 min, supernatant was aspirated, and cells were resuspended in complete medium. This wash step was repeated twice. Cells of both populations were mixed and seeded. 100 μM forskolin was added after two days and incubated for 24 h and 48 h. Live cell fluorescent microscopy was performed every 24 h (day 1-4), DiI has its emission maxima at 565 nm and was detected with a filter for red fluorescence, DiO has its emission maxima at 501 nm and was detected with a filter for green fluorescence. When cells fuse, they exhibit fluorescence at both wavelengths (565 and 501 nm) which is visualized by a yellow staining.

Caspase-3/7 staining

For fluorescent staining of activated caspase-3/7, CellEvent™ Caspase-3/7 Green ReadyProbes™ Reagent (Molecular Probes®, Life Technologies) was used. The principle of this assay is a nucleic acid binding dye which is conjugated to a DEVD-peptide. This dye is not fluorescent when the DEVD-peptide is conjugated and the complex is not bound to DNA. Activation of caspase-3/7 leads to cleavage of the DEVD, the dye binds to nucleic acid and becomes fluorescent.

BeWo cells were seeded in 6- or 24-well plates and treated according to the experimental protocol. On the day of detection of activated caspase-3/7, two drops of CellEvent™ Caspase-3/7 Green ReadyProbes™ Reagent per mL of medium were incubated for 30 min at 37°C. After this incubation, 2 drops of NucBlue® Live ReadyProbes™ Reagent (Molecular Probes®, Life Technologies) per mL of medium were added to stain all nuclei and incubated for 15 min at room temperature. Images were taken using a fluorescence microscope.

2.5.2 Immunohistochemistry

Tissue Sectioning

Placental explant tissue embedded in wax after being parafinized was cut into 3 μm thick sections using a rotary microtome. The sections were then picked up with a microscope slide from a water bath at 45°C and drained in an incubator at 65°C for a minimum of 60 minutes.

Dewaxing

Before starting the immunocytochemical protocols, the sections were dewaxed with two incubation steps in xylene for 2-3 minutes, followed by incubation steps with 100% ethanol (twice), 90% ethanol and distilled water for 2-3 minutes each.

Antigen Retrieval

A PickCell antigen retrieval unit was used in order to recover the protein epitopes of the cut sections. Briefly, the slides were placed into the Retriever Slide Chamber containing 70 mL of buffer A (pH 6) or B (pH 8). The Slide Chambers were placed in a rack and the retriever unit was filled with 200 mL of deionized water. After closing the retriever unit, a programme of 2:20 hours was started with 20 minutes of a heating process under pressure and 2 hours of a cooling process.

Optimization of primary Antibodies

For every antibody, the antigen retrieval was optimised by testing different buffers during the antigen retrieval process. For the antibodies M30 (M30 CytoDeath mouse monoclonal antibody, Roche), Ki67 (anti-Ki67 antibody (clone MIB-1), DAKO) and 11β-HSD2 (H-145, Santa Cruz), buffer A (pH 6) and buffer B (pH 8) were tested and buffer A gave stronger staining results for all antibodies. Furthermore, the sections were stained at a range of serial dilutions of the antibodies in order to determine the best antibody dilution for following stainings (Figure 2.8 for staining of human bowel (as a positive control tissue) and placental explant with a serial dilution of the M30 antibody). Optimal primary antibody dilution for M30 was 1:160, for Ki67 1:200 and for 11β-HSD2 1:300.

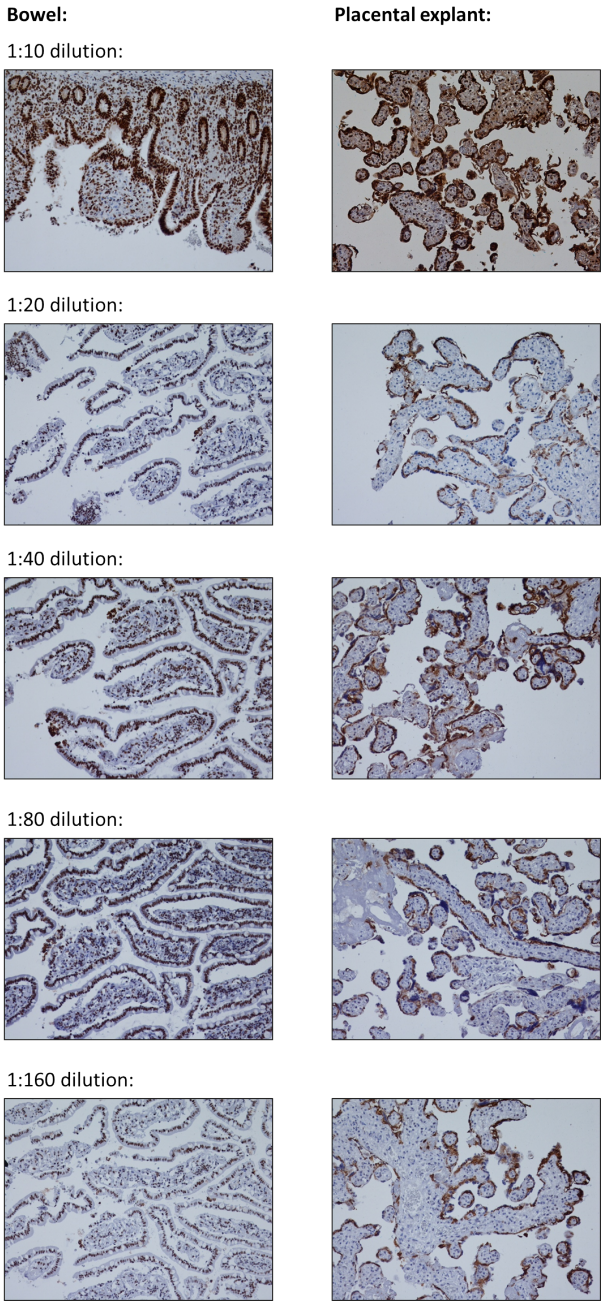

Figure 2.8: **IHC with M30 antibody (serial dilution).** Human bowel (as a positive control tissue) and placental explant were stained with different concentrations of the M30 antibody (weak staining in 1:20 dilution because the antibody solution did not cover the slide completely) to determine the optimal dilution for the following experiments. Representative images are shown, blue staining = haematoxylin staining, brown staining = M30 staining.

Immunostaining with Novolink Polymer Detection Kit

The Novolink Polymer Detection Kit (Leica) was used for performing the immunostaining and all described solutions were part of the kit unless otherwise stated. Following antigen retrieval, the sections were washed with distilled water and the borders around the tissue were marked with a PAP (hydrophobic) pen. After one washing step with TBST for 5 minutes, the endogenous peroxidase activity was blocked with the peroxidase block solution (2 drops = approximately 100 μL) for 5 minutes. Then after two washing steps with TBST for 5 minutes, the sections were incubated with 2 drops of protein block solution. After another two washing steps with TBST for 5 minutes, sections were incubated in primary antibody at optimal dilution (approximately 200 μL) overnight at 4°C. The next day, after two washing steps with TBST for 5 minutes, the sections were incubated in post primary block solution (2 drops) for 30 minutes. After further two washing steps with TBST for 5 minutes, sections were incubated with 2 drops of polymer solution for 30 minutes. Then, following two washing steps with TBST for 5 minutes, the sections were incubated with DAB working solution (1:20 dilution of DAB chromogen in DAB substrate) for 7 minutes. Before staining with haematoxylin solution for 1 minute, the sections were washed twice with distilled water. After a last wash with TBST for 5 minutes, the sections were dehydrated. Therefore, they were incubated each for 2-3 minutes in distilled water, 90% ethanol, 100% ethanol and xylene. Brightfield microscopy of the sections was carried out after mounting the slides.

2.6 ELISA

2.6.1 Single ELISA

BeWo cells and placental explants were cultured according to the experimental protocol. Supernatant was centrifuged (500 g, 5 min) before measuring the concentrations of hCG, progesterone, estradiol and cortisol using electrochemiluminescence immunoassays (Elecsys® ECLIA Kits, Roche Diagnostics). The measurements were carried out by the Department of Biochemistry (University Hospitals Coventry and Warwickshire NHS Trust).

2.6.2 Multiplex ELISA

Multiplex ELISA was carried out using the MSD Multiplex Apoptosis Panel Whole Cell Lysate Kit (Meso Scale Discovery) and the MSD Sector Imager 6000 (Meso

Scale Discovery). This ELISA technique uses a sandwich assay principle: capture antibodies against cleaved PARP, cleaved caspase-3, total p53 and phospho-p53 are coated on an electrode surface of a 96-well plate, binding of sample molecules to the capture antibodies occurs, and the SULFO-TAG labelled detection antibody binds to the sample molecules. After adding the provided read buffer, signals are detected as emitted light by the SULFO-TAG labels after voltage is applied to the plate electrodes (electrochemiluminescent detection).

Samples from placental explants were mechanically lysed in RIPA buffer (Santa Cruz) containing 10μL protease-inhibitor-cocktail (PIC), 10 μL PMSF, 10 μL orthovanadate (components of RIPA buffer kit) and 1:100-diluted phosphatase-inhibitor I and II (Sigma). After measuring the protein concentration using the BCA assay (Pierce) (see Chapter 2.4.2), samples were diluted in the above described lysis buffer with 0.1% Blocker A (part of the MSD Multiplex Apoptosis Panel Whole Cell Lysis Kit) to get a concentration of 16 μg protein per well (this correlates to a concentration of 0.64 μg/μL because 25 μL of sample were used in one well).

All materials described below are part of the MSD Multiplex Apoptosis Panel Whole Cell Lysate Kit (cleaved PARP, total p53, phospho-p53, cleaved caspase-3) and were made and diluted according to the manufacturer's instructions. The coated 96-well plate was washed once with 1x Tris Wash Buffer (=TWB) (prepared with deionized water) and then blocked with 150 μL of 3% blocking solution-A (diluted in 1x TWB), sealed, for 1 h at room temperature (=RT) with shaking. After 3 washing steps with 150 μL 1x TWB, 25 μL of 1% blocking solution-B (diluted in 1x TWB) was added to each well together with 25 μL of cell lysate and subsequently the plate was sealed and incubated for 1 h (during initial measurement) or 3 h (subsequent measurements) at RT with shaking. This incubation time was increased to augment the possibility to detect changes for p-p53 and t-p53 as increasing the total protein level was limited. After another 3 washing steps with 150 μL 1x TWB, 25 μL of detection antibody (diluted in 1x TWB with 1% Blocker A and 10 μL/mL Blocker D-R) was added to each well and then the plate was sealed and incubated for 1 h (during initial measurement) or 2 h (subsequent measurements) at RT with shaking. This incubation time was increased to augment the possibility to detect changes for p-p53 and t-p53. 150 μL 1x read buffer T (diluted in distilled water) was added to each well after 3 wash steps with 150 μL 1x TWB and then the plate was measured with the MSD Sector Imager 6000 (Meso Scale Discovery) immediately.

Determination of necessary Protein Concentration for Multiplex ELISA

An initial experiment using two placental explant samples (control or 10 μM staurosporine treated, cultured for five days with daily treatment) was performed in order to determine the protein concentration which is necessary for detecting changes in four molecules involved in apoptosis, namely cleaved caspase-3, cleaved PARP, total p53 and phosphorylated p53. Dilutions of the two samples (protein concentrations between 0.5 and 32 μg protein per well) were measured to test the signal intensity for cleaved caspase-3, cleaved PARP, total p53 and phosphorylated p53. Figure 2.9 A and B show that a measurable difference (>2 fold) in signal for cleaved caspase-3 between control and staurosporine-treated sample could be detected at all protein concentration (max. 3.9-fold increase of cleaved caspase-3) and for cleaved PARP at 32 μg protein per well (2.6-fold increase of cleaved PARP). Figure 2.9 C and D show that no difference between the two samples could be detected for total p53 and phosphorylated p53 over the range of protein concentrations tested. In subsequent experiments, a concentration of 16 μg protein per well was chosen because this was the highest concentration available to perform all assays. To compensate for the protein concentration available, the two incubation steps were increased from 1 h to 3 h as mentioned above.

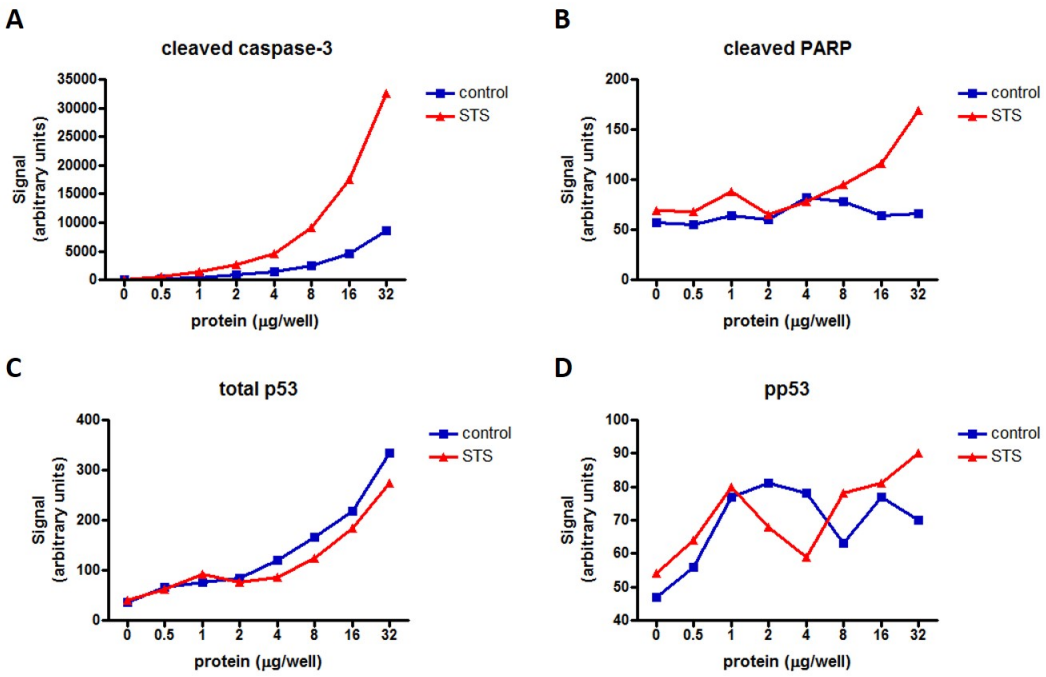

Figure 2.9: **Determination of necessary protein concentration for multiplex ELISA.** Analysis of the signal intensity for cleaved caspase-3, cleaved PARP, total p53 and phosphorylated p53 (pp53) at different protein concentrations from a control and a staurosporine (STS)-treated placental explant sample, n=1. Signals were detected using the multiplex ELISA system "'MSD Multiplex Apoptosis Panel Whole Cell Lysis Kit"'.

2.7 Viability and Apoptosis Assays

2.7.1 CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS)

To measure cell viability, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) was used. The principle of the assay is the reduction of the MTS tetrazolium compound to the coloured formazan product. Living cells produce NADPH and NADH which is required in the conversion of MTS into formazan and so the measured signal at 490 nm is proportional to the number of living cells [Cory et al., 1991; Berridge and Tan, 1993].

BeWo cells were seeded in a 24-well plate and treated according to the experimental protocol. On the last day, medium was removed and 500 μL F12K/DMEM medium without phenol red (Life Technologies) was added to each well with 50 μL of MTS solution. After incubating for 2 h at 37°C, the plate was rocked, 100 μL of supernatant of each well was transfered into a 96-well plate and absorbance at 490 nm was measured.

2.7.2 CellTiter-Blue® Cell Viability Assay

To measure cell viability in combination with apoptosis, the CellTiter-Blue® Cell Viability Assay was used. The principle of the assay is the reduction of the compound resazurin into the fluorescent resorufin ($579_{Ex}/584_{Em}$) product by living cells [Gloeckner et al., 2001].

BeWo cells were seeded in a 24-well plate and treated according to the experimental protocol. On the last day, 50 μL of Cell Titer Blue solution was added to each well. After incubating for 2 h at 37°C, the plate was rocked, 100 μL of supernatant of each well was transfered into a 96-well plate and fluorescence at 590 nm was measured. Subsequently, a multiplex assay with the ApoONE® Homogeneous Caspase-3/7 Assay was possible (Chapter 2.7.3).

2.7.3 ApoONE® Homogeneous Caspase-3/7 Assay

To measure activated caspase-3/7, the ApoONE® Homogeneous Caspase-3/7 Assay was used. The principle of this manufactured Caspase-3/7 assay is the detection of a fluorescent cleaved substrate at 521 nm (= emission maximum). The activated caspase-3 and -7 cleave their substrates at the amino acid recognition site DEVD [Thornberry et al., 1997; Nicholson, 1999]. The profluorescent substrate (Z-DEVD-Rhodamine 110, provided by the kit) contains this DEVD amino acid sequence and

activated caspase-3 and -7 cleave this form and the fluorescent substrate Rhodamine 110 is generated.

BeWo cells were seeded in a 24-well plate and treated according to the experimental protocol. On the last day, 100 μL of ApoONE solution (1 μL substrate in 1 mL buffer, substrate and buffer were components of the kit) was added to each well. After incubating for 4 h at room temperature, the plate was rocked, 200 μL of supernatant of each well was transfered into a black 96-well plate and fluorescence at 530 nm was measured.

As a positive control experiment, BeWo cell were treated with 1 μM staurosporine (which induces caspase-3/7 activation) for 4 h before the ApoONE was added. Furthermore, background fluorescence was determined by adding ApoONE to F12K medium (without cells). Figure 2.10 shows that background fluorescence was minimal with 51 arbitrary units and that staurosporine activated caspase-3/7 significantly.

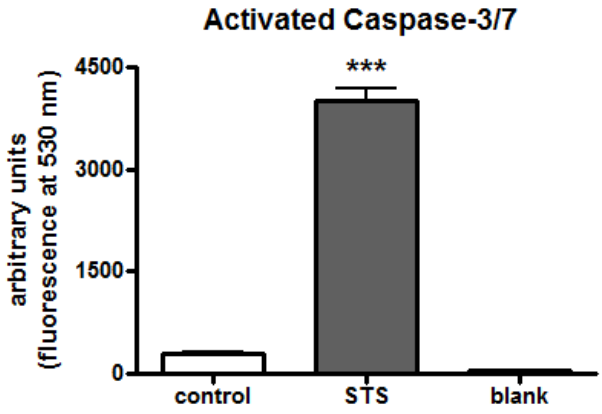

Figure 2.10: **Activation of caspase-3/7 in BeWo cells by staurosporine.** BeWo cells were treated with 1 μM staurosporine (STS) for 4 h before the ApoONE assay was performed. n=3, t-test: $p<0.001$ (=***).

2.8 Statistical Analysis

Data were analyzed and graphs were prepared using the GraphPad Prism 4 software. Experiments were repeated independently at least three times to allow statistical analysis. Depending on the design of the experiment, comparisons among treatment groups were made using Student's t-test (when comparing two groups), 1-way or 2-way ANOVA (when comparing more than two groups) to test for statistical significance. In case of ANOVA analysis, Bonferroni post-test analysis was applied to perform multiple comparison tests. The mentioned parametric tests are based on the assumption of normally distributed data. To fulfill this requirement, data of qRT-PCR and percentage data was log-transformed prior to analysis. However, tests for normality could not be carried out as the number of experiments was too small. To perform parametric tests with small sample numbers, it is important that the measured samples are representative of the population. Small sample size is only reasonable under the assumption that the variability within the population is small. As data from cell lines can be considered to have a low variation between the samples, parametric tests were used for analysis in this thesis. Furthermore, data from placental explants were analyzed with parametric tests as the tissue was collected from healthy patients (and not from patients with a pathological condition whose tissue can be expected to give data with a greater variation between the samples).

Chapter 3

Effects of CRH and LPS on Hormone Secretion and Cell Turnover of Placental Explants

The placental explant culture is a suitable model for the analysis of hCG secretion by the syncytiotrophoblast and its characteristics are described in this chapter. Furthermore, the responsiveness of the placental explant tissue (for details see chapter 2.1.1) to external stimuli was investigated. The hormone CRH is of particular interest because it is elevated in placenta-related diseases [Laatikainen et al., 1991; Goland et al., 1993; Warren et al., 1992]. To further investigate whether an inflammatory environment changes the effects of CRH, LPS was used as another external stimuli. Inflammatory environments are often observed in placental pathologies such as pre-eclampsia (Chapter 1.3.1.1) and placental infection, in maternal pathologies such as diabetes and obesity (Chapter 1.3.2) as well as in the physiological parturition process (Figure 1.17). LPS binds to the toll-like receptor 4 (= TLR4) which has been shown to be expressed in the placenta in all trimesters [Holmlund et al., 2002; Beijar et al., 2006]. Holmlund et al. [2002] further showed that treatment of placental explants with LPS leads to an induction of IL-6 and IL-8 production, suggesting the presence of a functional TLR 4 in the placenta and a signalling inflammatory response via the TLR4-NFκB pathway.

3.1 Detection of Proliferation in Placental Explants

For the detection of proliferation in placental explants, immunohistochemical Ki67 staining was performed (see chapter 2.5.2 for experimental details). The Ki67 antibody stains proliferating cells by binding to a nuclear antigen which is only present in proliferating cells [Gerdes et al., 1983].

As an initial experiment, freshly isolated placental explant tissue and human tonsil (positive control for Ki67 antibody) were stained for Ki67. Figure 3.1 A shows that the Ki67 staining was carried out successfully since the tonsil shows strong Ki67 staining. Also the placental tissue showed that a few trophoblast nuclei were positively stained for Ki67 (Figure 3.1 C, D). The negative control did not show any detectable Ki67 staining (Figure 3.1 B).

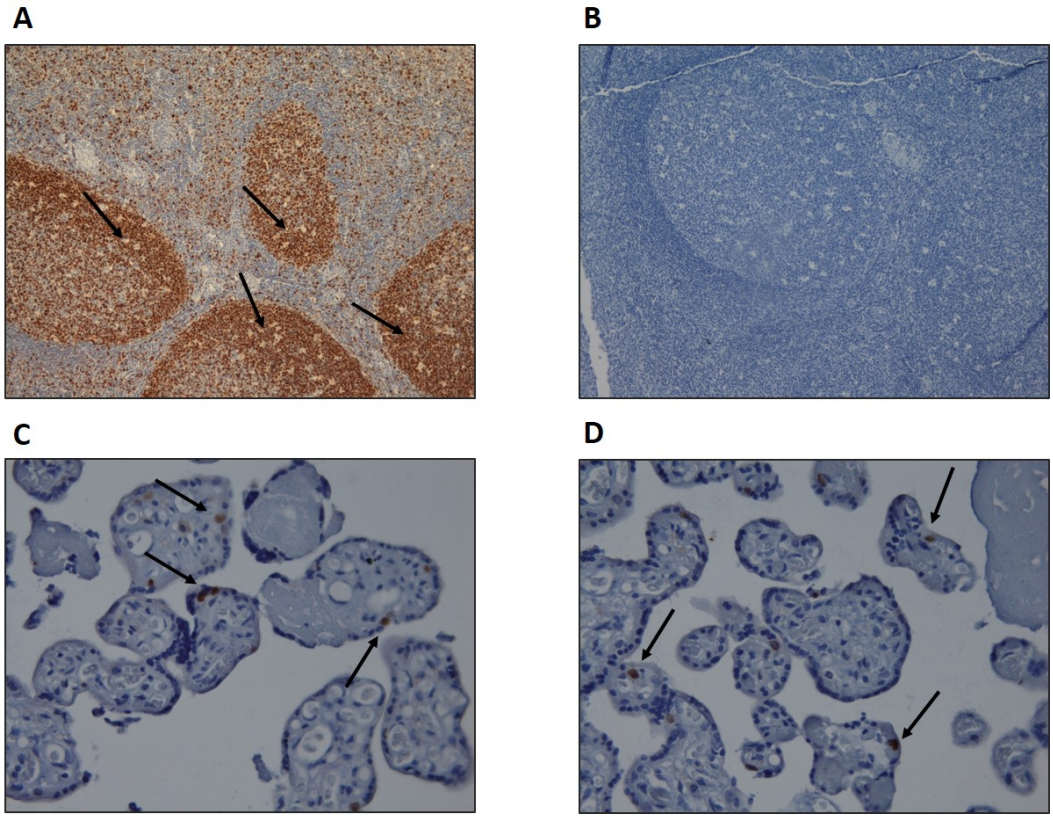

Figure 3.1: **Proliferation in placental tissue.** Ki67-staining of **(A)** human tonsil (= positive control tissue for the Ki67 antibody), **(B)** human tonsil (= negative control, no Ki67 antibody), **(C, D)** placental explant tissue. **A, B:** 20x objective, **C, D:** 40x objective. n = 2 placentae, representative images are shown. Blue staining = haematoxylin staining. Arrows indicate brown staining from the Ki67-staining.

Responsiveness of Placental Explant Proliferation to CRH and LPS

To investigate whether CRH and/or LPS alter placental proliferation, placental explants were treated with 1 μM CRH and/or 10 μg/mL LPS for five days (see chapter 2.2 for treatment protocol, CRH and LPS were replenished every 24 h). Subsequently, the tissue was processed for the immunohistochemical detection of Ki67.

Qualitative assessment showed that the different treatments with 1 μM CRH and 10 μg/mL LPS did not show any detectable difference in cell proliferation of placental explants after five days of treatment (Figure 3.2). An experienced histochemistry-biomedical scientist confirmed this and the samples were not processed for quantitative assessment.

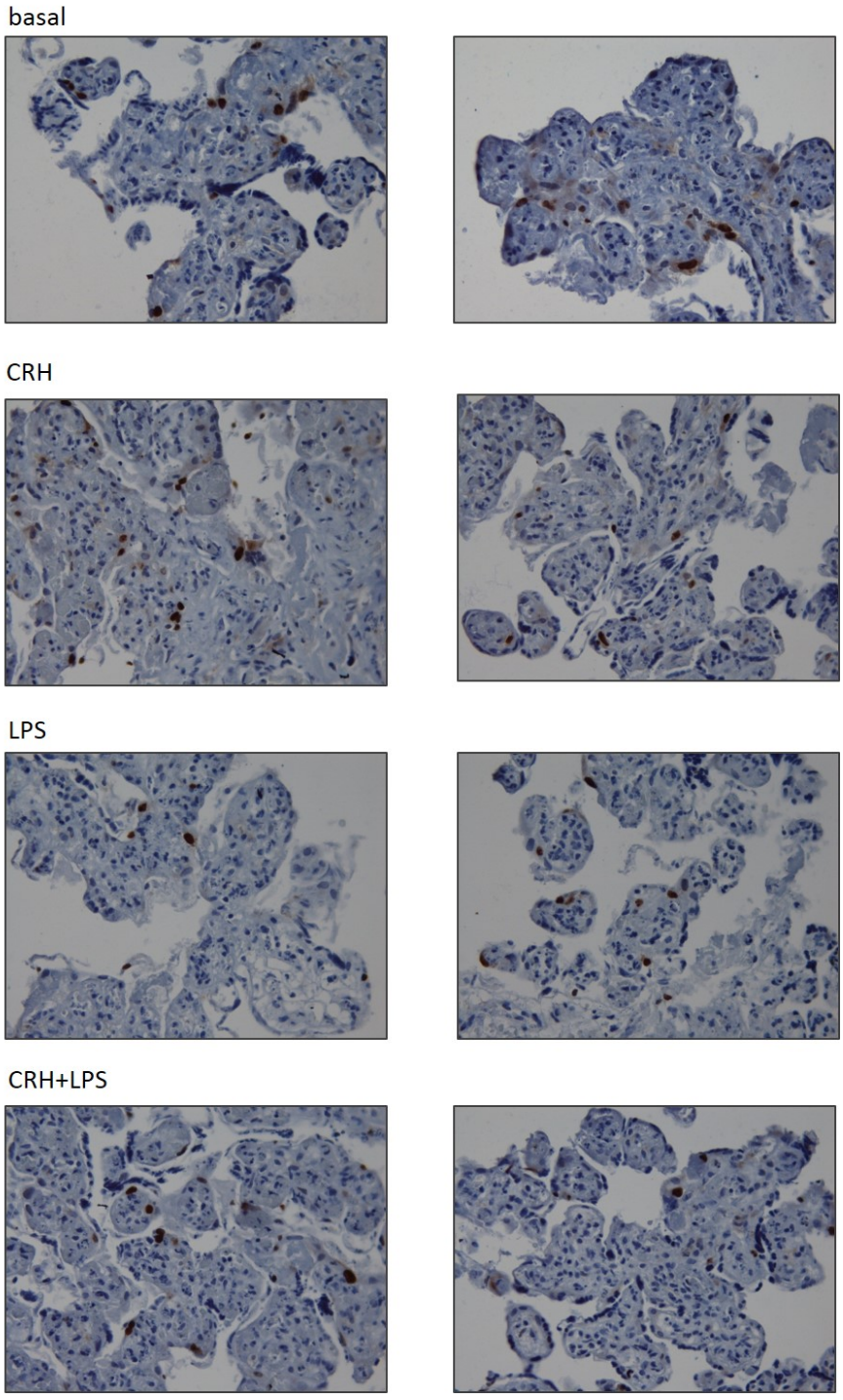

Figure 3.2: **Effect of CRH and LPS on placental explant proliferation.** Ki67 staining of placental explants after being cultured for five days and treated with 1 μM CRH and/or 10 μg/mL LPS. 40x objective, n = 2 placentae, representative images are shown. Blue staining = haematoxylin staining, brown staining = Ki67-staining.

3.2 Measurement of secreted Hormones and its Responsiveness to CRH and LPS

Placental explants were treated for 6 days with 1 μM CRH and/or 10 μg/mL LPS and the hCG release in the supernatant was assessed every 24 h. On day 6, the explants from each well were collected to determine the protein concentration (see Chapter 2.1.1 for experimental details).

3.2.1 CRH and LPS Effects on hCG Secretion

Simán et al. [2001] described that cultured placental explants lose their syncytiotrophoblast layer after the first day of culture and that a new functional syncytiotrophoblast layer is regenerated by day 4. During culture of placental explants, focussing on the basal treatment, a decrease in hCG secretion was observed in the first few days of culture by 80% when comparing hCG secretion from day 1 with day 2 (Figure 3.3 A) suggesting that the existing syncytiotrophoblast layer was lost at this stage of the experiment. On day 2 and 3 the minimal amount of hCG was measured in the supernatant (day 2: 59.7 mIU/mL $\pm$ 9.2 SEM, day 3: 62.2 mIU/mL $\pm$ 7.1 SEM). On day 4 the hCG secretion had started to increase, peaking at an hCG secretion of 107.7 mIU/mL $\pm$ 18.2 SEM on day 5 until it started to drop again on day 6. This observation suggests that a new syncytiotrophoblast layer was regenerated during this time which is in agreement with Simán et al. [2001].

This pattern of hCG release, namely the reduction in hCG release from day 1 to day 2, the plateau phase from day 2 to day 3 and the increase of hCG release characteristics from day 3 to day 6 peaking at day 5, was not altered in the presence of the four different treatments (basal, 1 μM CRH, 10 μg/mL LPS, 1 μM CRH + 10 μg/mL LPS, Figure 3.3 A).

However, Figure 3.3 B to E show the differences in the increase of hCG under the different treatments (B = basal, C = CRH, D = LPS, E = CRH+LPS) from day 2 to day 5 of explant culture. Under basal conditions, there was a significant increase of hCG secretion by 80% from day 2 to day 5 (Figure 3.3 B). CRH treatment led to a significant enhancement of hCG secretion by 160% during the same period (Figure 3.3 C). In contrast, LPS treatment did not increase hCG secretion between day 2 and day 5 (Figure 3.3 D). Simultaneous treatment of CRH and LPS led to a significant hCG increase by 78% (Figure 3.3 E) which is comparable to basal culture conditions (Figure 3.3 B).

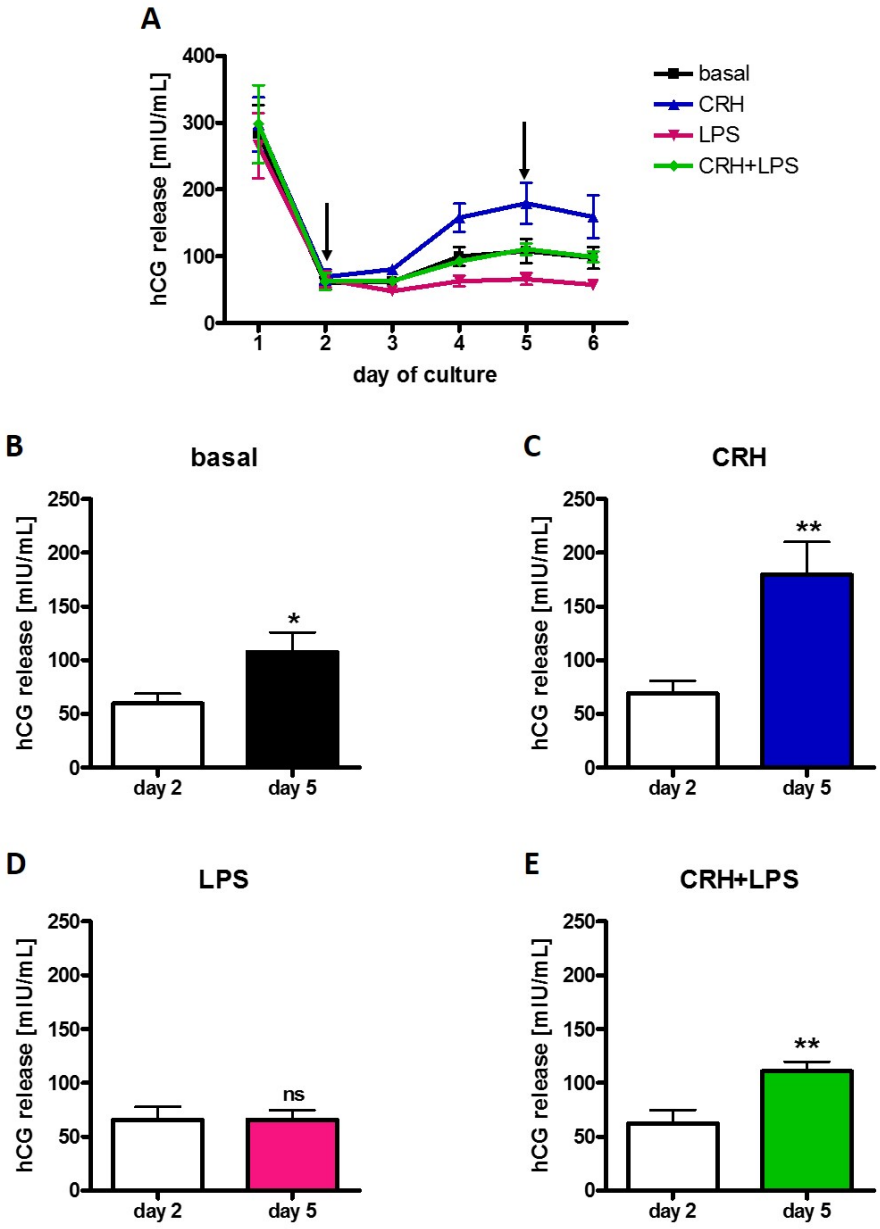

Figure 3.3: **Effect of CRH and LPS on hCG production of placental explants. (A)** Daily hCG release by cultured placental explants treated with 1 μM CRH, with and without 10 μg/mL LPS. **(B-E)** hCG secretion on day 2 and 5 under the different treatments. hCG concentration was normalized to protein concentration, n=3 different placentae (treatments in triplicates), data are expressed as mean value $\pm$ SEM, t-test: ns = non-significant, $p<0.05$ (=*), $p<0.01$ (=**).

3.2.2 Inconsistency of Placental Explants

Even though all of the placentae were obtained from healthy individuals, considerable differences in the hCG secretion levels as early as on day 1 was observed. Figure 3.4 shows the hCG secretion on day 1 from the placentae labelled as "'high hCG responders"' (data from these placentae were presented in Figure 3.3) which was significantly higher than the hCG secretion from placentae labelled as "'low hCG responders"'. During preparation of the low hCG responder explants, these placentae revealed different morphological characteristics such as a pale rose color, a lot of red blood cells in the wells during the whole period of the 6-day culture, white colored firm fragments in the soft placental tissue, and one patient had an elevated blood pressure.

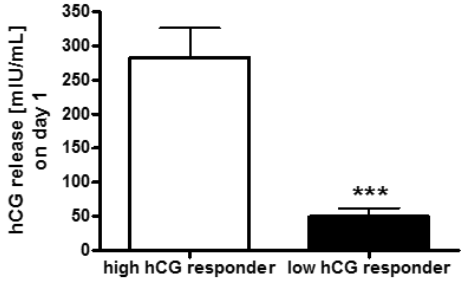

Figure 3.4: **hCG secretion on day 1 of placental explant culture.** hCG concentration was normalized to protein concentration, n=3 different placentae (treatments in triplicates), data are expressed as mean value ± SEM, t-test: $p<0.001$ (=***).

In these low hCG responding placentae, the responsiveness to treatments with CRH and LPS was different (Figure 3.5) compared to the high hCG responder placentae (previously shown in Figure 3.3). Figure 3.5 A shows that basal culture conditions without any stimuli led to the lowest hCG secretion between day 2 and day 6. Figure 3.5 B to E show that all culture conditions led to a significant increase of hCG secretion between day 2 and day 5. Under basal conditions, there was an increase of hCG secretion by 5.8-fold from day 2 to day 5 (Figure 3.3 B). CRH treatment further increased the hCG secretion by 9.1-fold from day 2 to day 5 (Figure 3.3 C). LPS treatment enhanced hCG secretion by 7.2-fold between day 2 and day 5 (Figure 3.3 D). Simultaneous treatment of CRH and LPS led to the highest increase of hCG secretion by 10.9-fold (Figure 3.3 E) suggesting a synergistic

effect of CRH and LPS on hCG secretion. It seemed that placentae with a low hCG secretion on day 1 of the explant culture were highly responsive to various stimuli. Because all of the placentae (high and low hCG responders) were obtained from individuals without any established or confirmed pathology, it was concluded that the considerable variation in responses is a major limitation of this experimental approach.

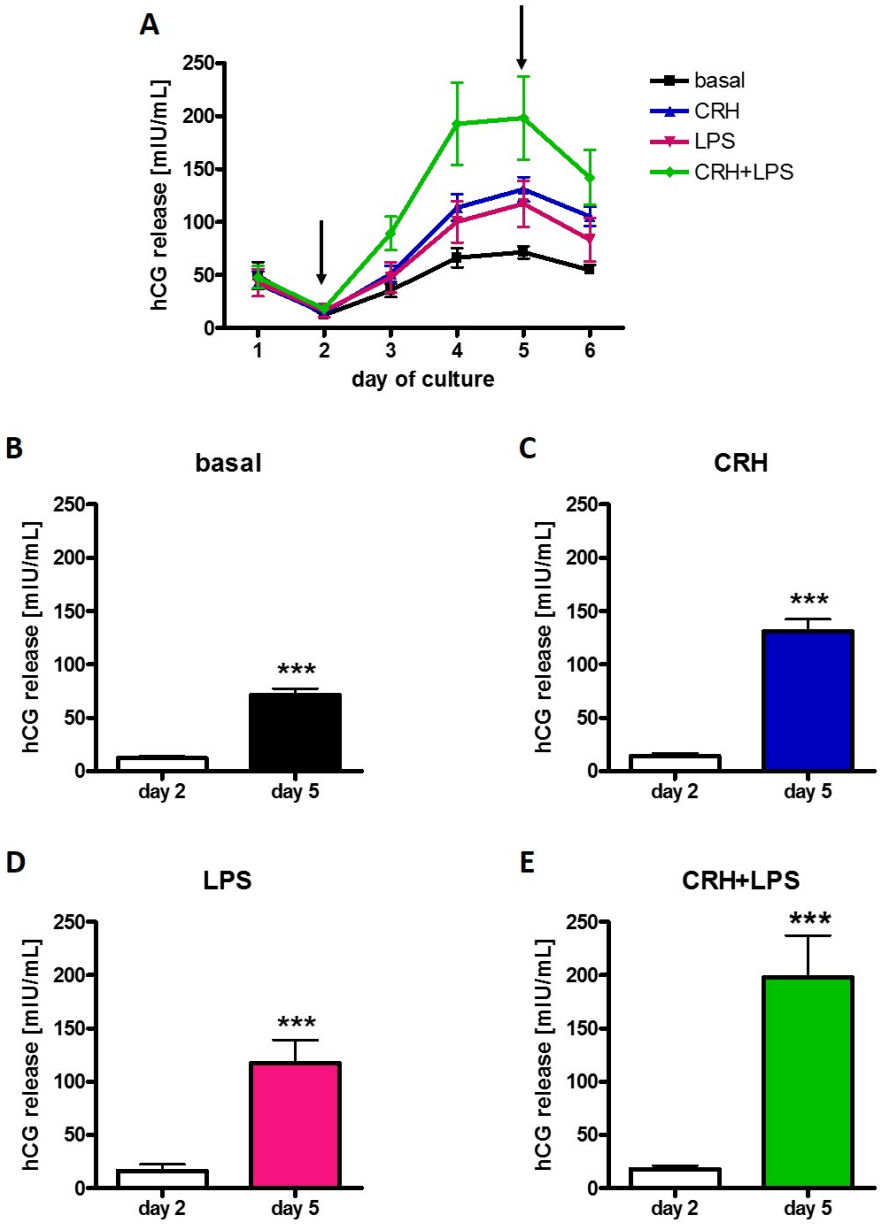

Figure 3.5: **Effect of CRH and LPS on hCG production of low hCG responder placentae. (A)** Daily hCG release by cultured placental explants treated with 1 μM CRH, with and without 10 μg/mL LPS. **(B-E)** hCG secretion on day 2 and 5 under the different treatments. hCG concentration was normalized to protein concentration, n=3 different placentae (treatments in triplicates), data are expressed as mean value $\pm$ SEM, t-test: $p<0.001$ (=***).

3.3 Detection of Apoptosis in Placental Explants

Detection of apoptosis in placental explants was performed both qualitatively and quantitatively after CRH and/or LPS treatment. Previous studies linked CRH to apoptotic processes and both a pro- and an anti-apoptotic role for CRH has been shown under different circumstances [Schmid et al., 2011; Fox et al., 1993; Facci et al., 2003; Dermitzaki et al., 2002; Minas et al., 2007]. For example, Schmid et al. [2011] shows an anti-apoptotic effect of CRH on apoptosis in rat INS-1 insulinoma cells and Fox et al. [1993] and Facci et al. [2003] could demonstrate a neuroprotective effect of CRH against apoptosis in brain slices and in cerebral granule neurons, whereas Dermitzaki et al. [2002] detected a pro-apoptotic effect of CRH in PC12 cells which involves the CRH-R1 signalling and Minas et al. [2007] shows that CRH induces apoptosis via Fas-activation in extravillous trophoblasts. As CRH is elevated in placental-related diseases, it was considered possible that CRH is also involved in apoptotic processes in cytotrophoblast cells or in the syncytiotrophoblast and this might affect hCG release.

As a qualitative method, an immunohistochemical staining (M30-staining) for cleaved cytokeratin-18, which is a substrate of activated caspase-3, was performed. The M30-staining is a specific staining for apoptotic trophoblasts [Kadyrov et al., 2001]. As the quantitative method, the apoptosis marker molecules cleaved caspase-3, cleaved PARP, p53 and phosphorylated p53 were measured with a multiplex ELISA technique.

3.3.1 Qualitative Detection of cleaved Cytokeratin-18

Induction of Apoptosis by Staurosporine

In order to evaluate the M30-staining, placental explants were treated with 10 μM staurosporine (STS) for five days (fresh STS was added every 24 h). Staurosporine inhibits the protein kinase-C, which leads to the activation of caspase-3 (=cleavage of procaspase-3 to caspase-3) [Tamaoki and Nakano, 1990; Jacobsen et al., 1996] and can therefore be used as a positive control to demonstrate activation of apoptosis mechanisms.

Indeed, staurosporine-treated placental explants were stained for cleaved cytokeratin-18 and they showed, as expected, a strong positive M30-staining (Figure 3.6).

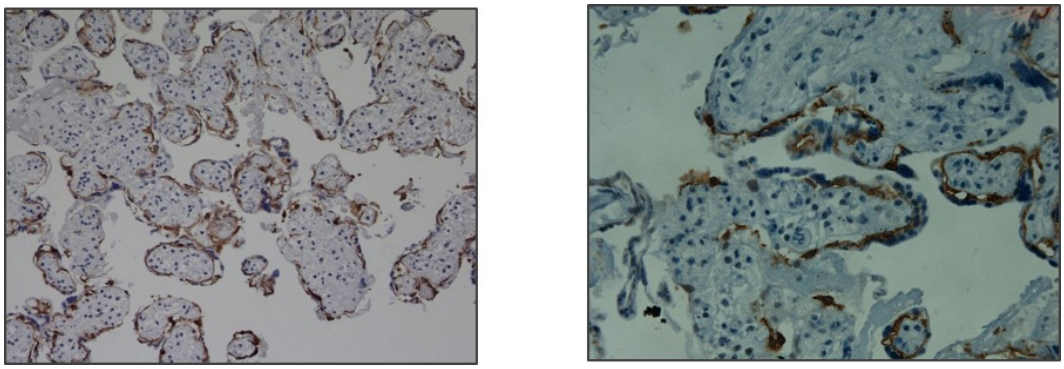

Figure 3.6: **Effect of staurosporine on placental explant proliferation.** M30-staining of staurosporine-treated placental explant. The explants were treated for five days with 10 μM staurosporine. Left: 20x objective, right: 40x objective. n = 3 placentae, representative images are shown. Blue staining = haematoxylin staining, brown staining = M30-staining.

Apoptosis of Fresh Placental Tissue

Fresh tissue from the placenta exhibited low apoptotic processes in the villous tissue as only minimal staining for cleaved cytokeratin-18 could be detected (Figure 3.7).

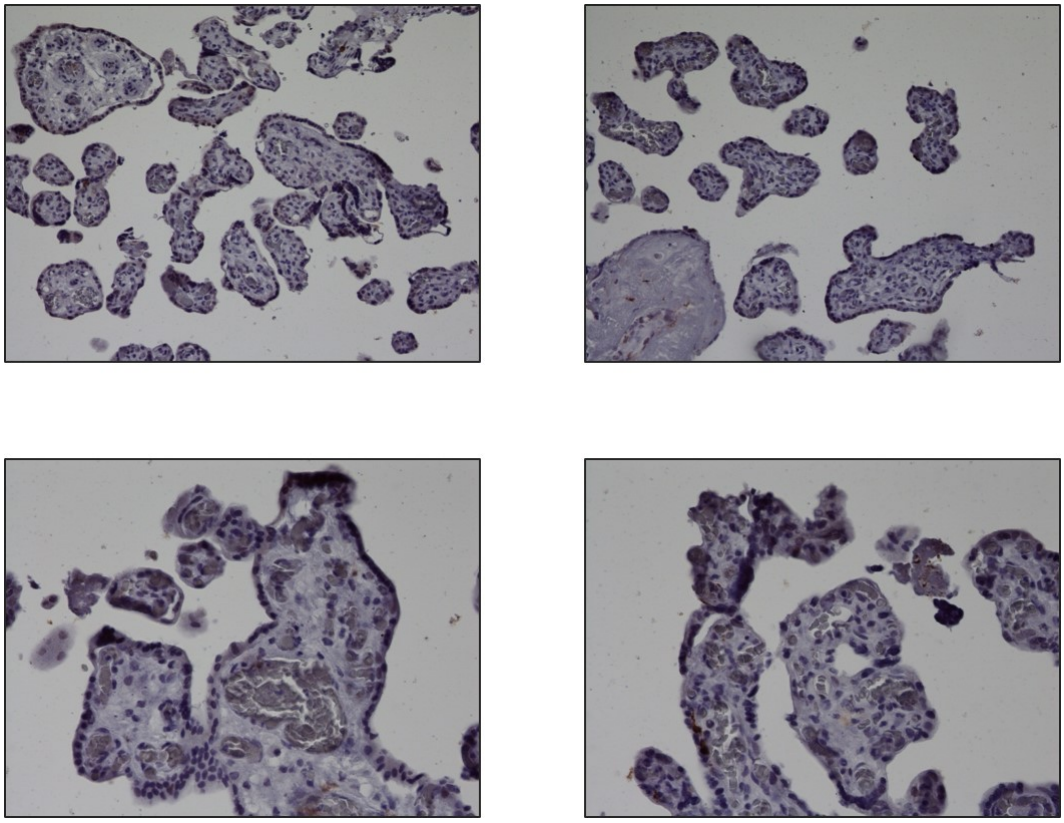

Figure 3.7: **Apoptosis in fresh placental explant tissue.** M30-staining of fresh placental tissue (= day 0). Neither cytotrophoblast areas nor syncytiotrophoblast areas showed positive staining for M30 (= no brown staining). Top: 20x objective, bottom: 40x objective. n = 2 placentae, representative images are shown. Blue staining = haematoxylin staining.

3.3.2 CRH and LPS Effects on Apoptosis in placental Explants (qualitative)

M30-staining of placental explant sections, which were cultured and treated with 1 μM CRH and/or 10 μg/mL LPS for five days, showed a positive staining for cleaved cytokeratin-18 (Figure 3.8 and 3.9). Qualitative assessment indicated that the different treatments (control, CRH, LPS, CRH+LPS) showed a positive staining in the same amount (Figure 3.8). In contrast, treatment with CRH in another placental explant experiment showed a positive M30-staining to a lesser degree compared to the control tissue (Figure 3.9).

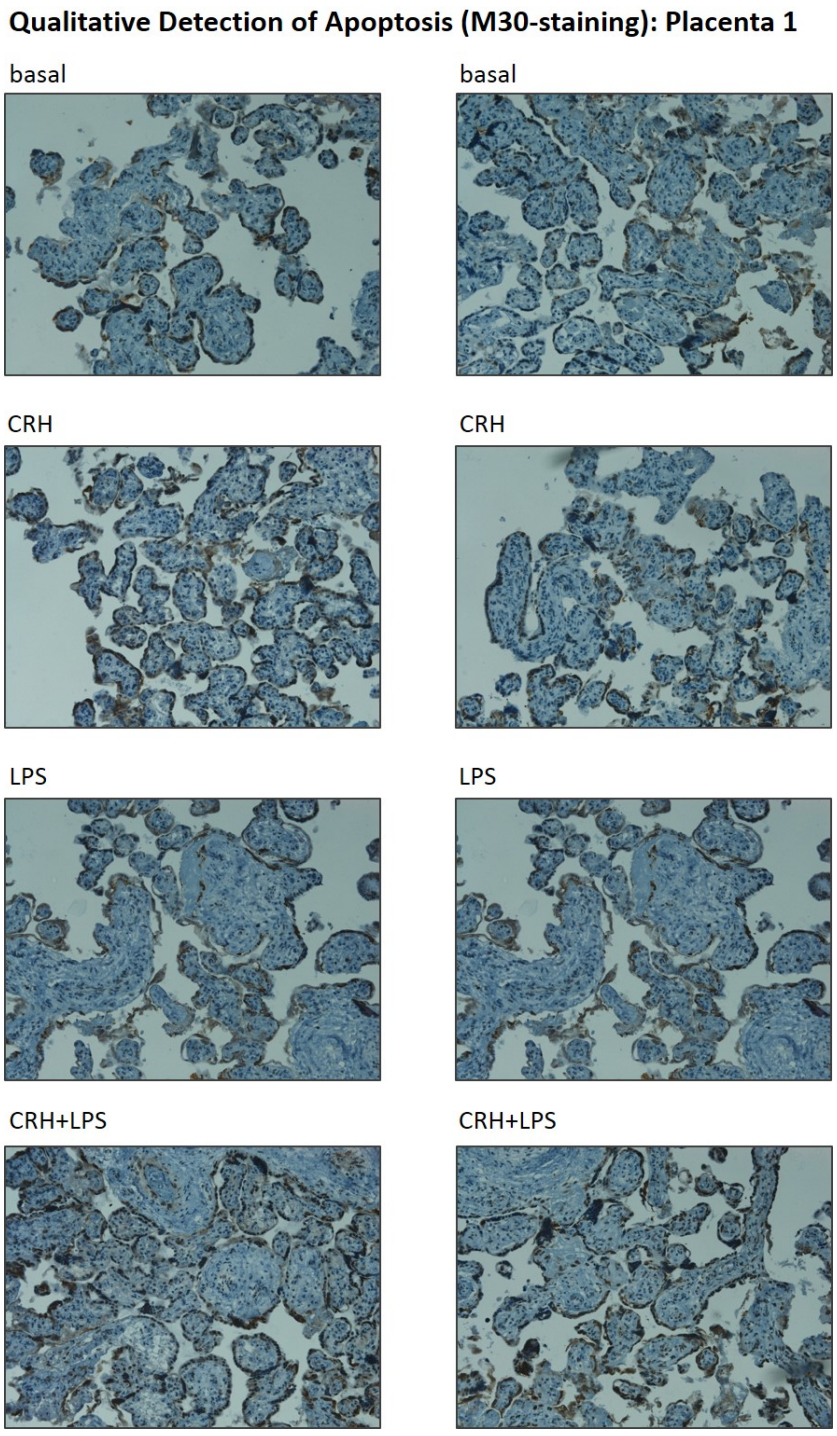

Figure 3.8: **Effect of CRH and LPS on placental explant apoptosis.** M30-staining of placental explants after treatment with 1 μM CRH and/or 10 μg/mL LPS for five days. 20x objective. n = 2 placentae, representative images are shown. Blue staining = haematoxylin staining, brown staining = M30-staining.

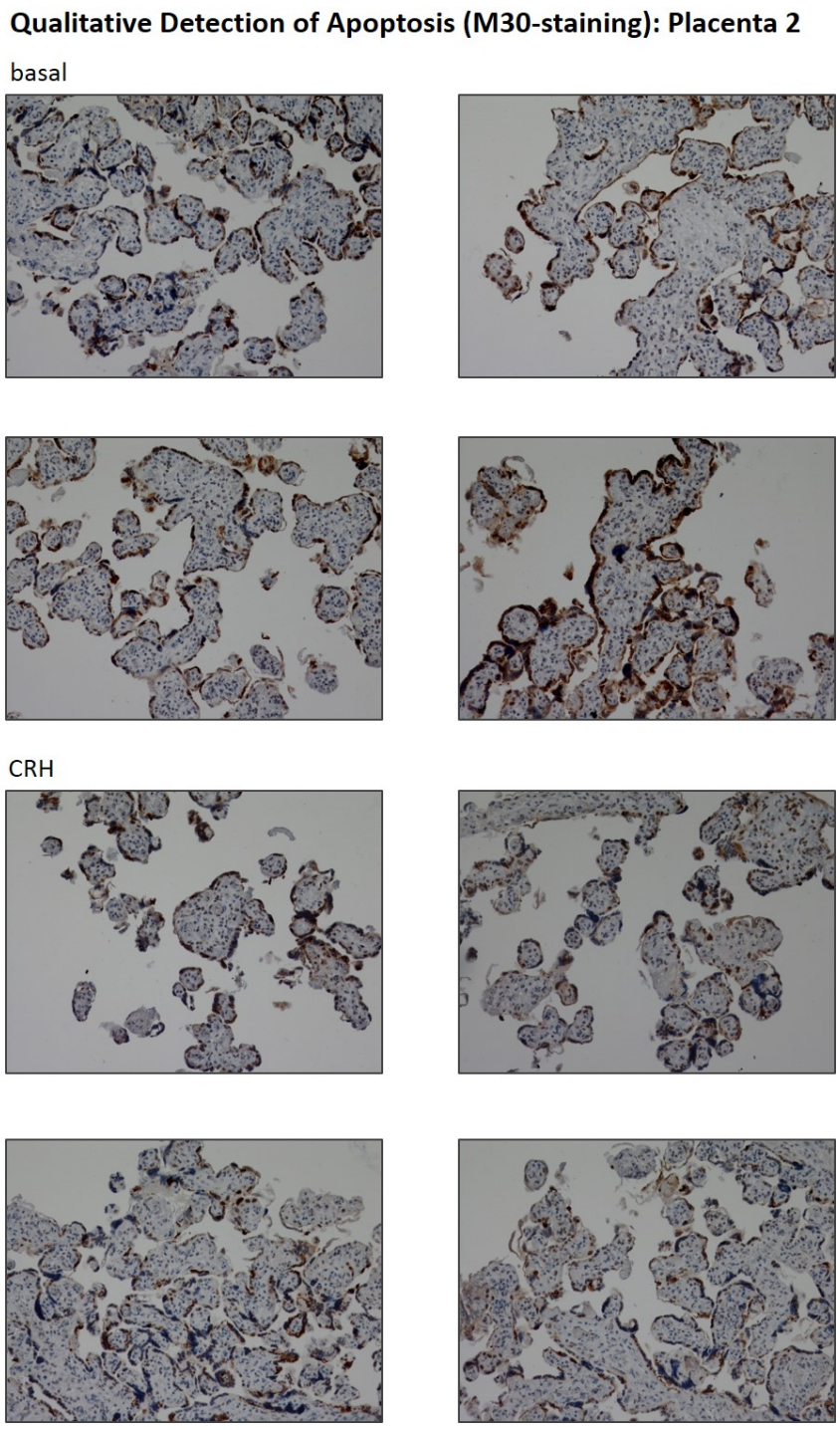

Figure 3.9: **Effect of CRH on placental explant apoptosis.** M30-staining of placental explants after treatment with 1 μM CRH for five days. 20x objective. n = 2 placentae, representative images are shown. Blue staining = haematoxylin staining, brown staining = M30-staining.

3.3.3 Quantitative Detection of four Marker Molecules of Apoptosis

Since the M30-staining is a qualitative method to detect apoptosis, an ELISA technique allowing a more precise quantitative measurement was performed to measure apoptotic marker molecules.

Placental explants were cultured for four and five days before the tissue was lysed and molecules involved in apoptosis (cleaved caspase-3, cleaved PARP, total p53 and phosphorylated p53) were measured with the multiplex ELISA technology (for methodological details see Chapter 2.6.2). Additionally, an internal control from day 0 was taken to be able to determine the initial apoptosis status in the tissue before starting the treatments.

In later stages of the apoptotic process, effector procaspases such as procaspase-3 are being activated (cleaved) by initiator caspases (caspase-8 and -9) (Figure 1.13). These activated forms are called (cleaved) caspases and they have proteolytic activity and cleave many substrates including PARP (= poly-(ADP-ribose)-polymerase). The apoptosis can be initiated by the extrinsic or intrinsic pathway (Chapter 1.2.6.1). The molecule p53 can activate apoptosis via the intrinsic pathway as p53 has been shown to upregulate the proapoptotic member Bax of the Bcl-2 family, the proapoptotic factor PUMA (= p53 upregulated modulator of apoptosis) and Apaf-1 (= apoptotic protease activating factor-1) which is a component of the apoptosome [Miyashita and Reed, 1995; Nakano and Vousden, 2001; Moroni et al., 2001]. Also, p53 can interact with the anti-apoptotic members Bcl-2 and Bcl-X_L of the Bcl-2 family and is thereby involved in the regulation of apoptosis [Vaseva and Moll, 2009].

Apoptosis under basal Placental Culture Condition

In order to determine whether the culture conditions led to an increase in apoptosis, we cultured placental explants for four and five days with daily medium change. Figure 3.10 shows that culturing of placental explants for several days led to a 5.9-fold and 4.8-fold increase in cleaved caspase-3 expression after four and five days of culture compared to day 0 cleaved caspase-3 levels. Cleaved PARP, total p53 and phosphorylated p53 expression did not increase after several days of culture. The result suggests that possibly the apoptotic cascade is stopped because cleavage of the caspase-3 substrate PARP did not occur.

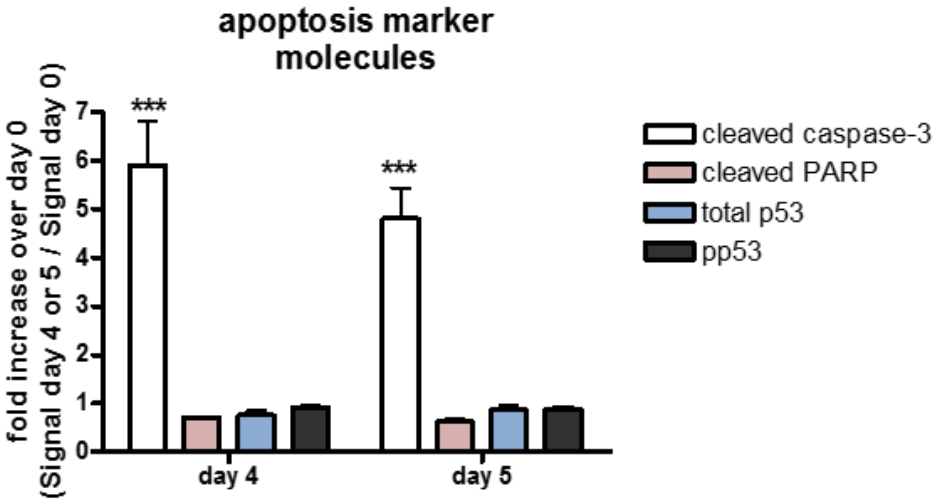

Figure 3.10: **Activation of apoptotic marker molecules after 4 and 5 days of basal placental explant culture.** Quantitative analysis of cleaved caspase-3, cleaved PARP, total p53 and phosphorylated p53 expression in placental explants after four and five days of culture compared to day 0. Results are from n = 3 placentae (treatments in duplicates), data are expressed as mean value ± SEM, t-test: $p<0.001$ (=***) comparison of day 4/5 with day 0 for every marker molecule.

3.3.4 CRH and LPS Effects on Placental Explants (quantitative)

Subsequent experiments investigated whether CRH and/or LPS treatments of placental explants altered expression of molecules of apoptosis (cleaved caspase-3, cleaved PARP, total p53 and phosphorylated p53). As described, the explants were treated every day with either 1 μM CRH, or 10 μg/mL LPS, or 1 μM CRH + 10 μg/mL LPS. Figure 3.11 shows that none of the treatments led to a significantly altered expression of cleaved caspase-3, cleaved PARP, total p53 and phosphorylated p53 after four or five days of culture.

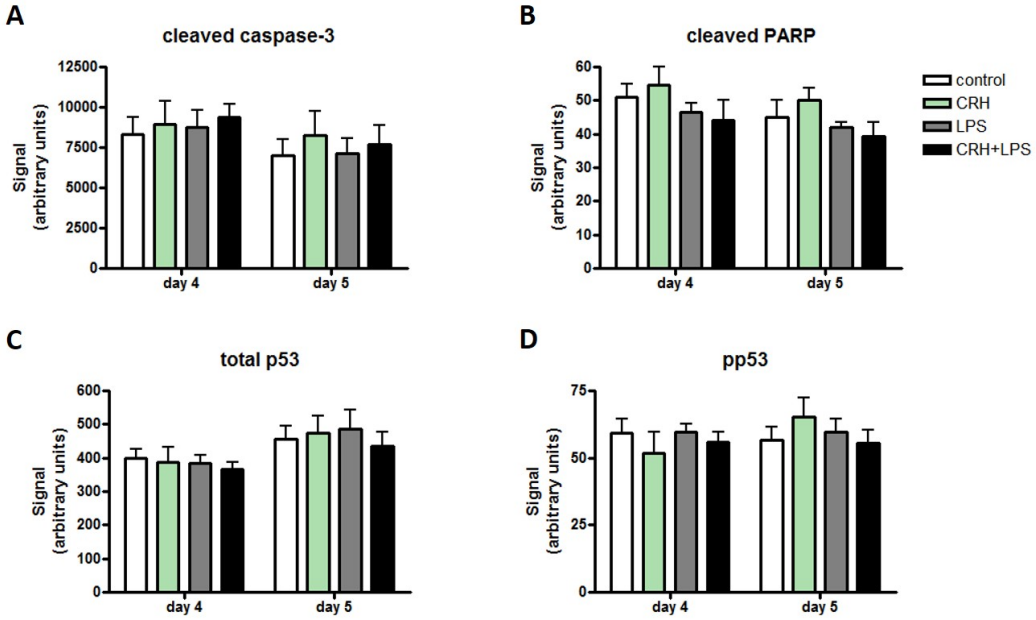

Figure 3.11: **Effect of CRH and LPS on activation of apoptotic marker molecules.** Quantitative analysis of cleaved caspase-3 **(A)**, cleaved PARP **(B)**, total p53 **(C)** and phosphorylated p53 **(D)** expression in placental explants following treatment with 1 μM CRH, 10 μg/mL LPS or 1 μM CRH + 10 μg/mL LPS for four and five days. Results are from n = 3 placentae (treatments in duplicates), data are expressed as mean value ± SEM, 2ANOVA with Bonferroni post test: no significances. Y-axis: Signal is shown in arbitrary units.

3.4 Initial Characterization of Proteins involved in GC Metabolism in Placental Explants

3.4.1 Detection of 11β-HSD2 and P-glycoprotein (ABCB1) mRNA Expression in Placental Explants

In an initial experiment, mRNA expression of 11β-HSD2 and P-glycoprotein, which are both components of the placental glucocorticoid barrier, was determined to validate the qRT-PCR method in the placental explant system. As a positive control, placental explants were treated with 1 μM Dexamethasone (Dex), which leads to induction of glucocorticoid-responsive genes, every 24 h for five days of culture. Figure 3.12 A and B show that 1 μM Dex highly up-regulated 11β-HSD2 and P-gp mRNA expression. The triplicates shown derive from three different wells with placental explants isolated from one placenta and the relative substantial differences in the expression of 11β-HSD2 and P-glycoprotein mRNA suggest intravariability in the placental explant system. Intravariability describes the variability between samples collected from one placenta. This intravariability led to a revised protocol focusing on protein expression in the subsequent experiments.

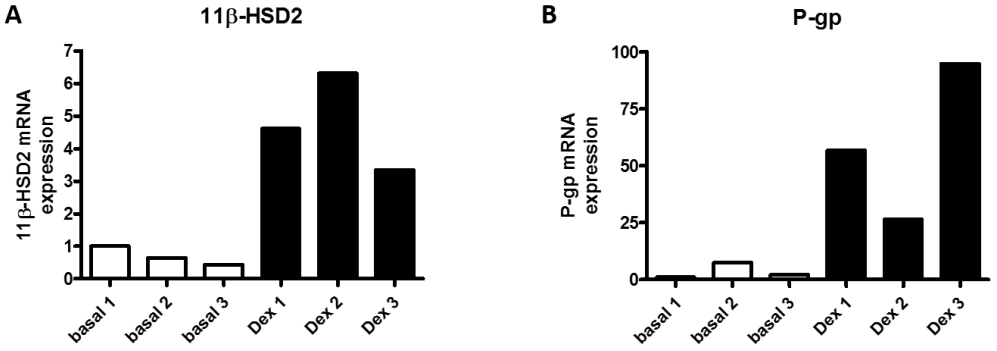

Figure 3.12: **mRNA expression of 11β-HSD2 and P-gp in placental explants.** qRT-PCR result of a pilot experiment. Placental explants were cultured for five days with 1 μM Dex before mRNA was isolated and qRT-PCR for **(A)** 11β-HSD2 and **(B)** P-gp was performed. n = 1 placenta (triplicates for each treatment). qRT-PCR data was normalized to 18rRNA.

3.4.2 Detection of 11β-HSD2 Protein in Placental Explants

As a positive control tissue for immunohistochemical 11β-HSD2 staining human bowel was used and indeed it displayed a strong staining for 11β-HSD2 (Figure 3.13 A). Staining was specific since the negative control did not show a brown staining (Figure 3.13 B). The placental explant which was cultured for five days under basal conditions displayed a strong staining for 11β-HSD2 in the syncytiotrophoblast layer and some of the trophoblast cells had a weak staining for 11β-HSD2 as well (Figure 3.13 C).

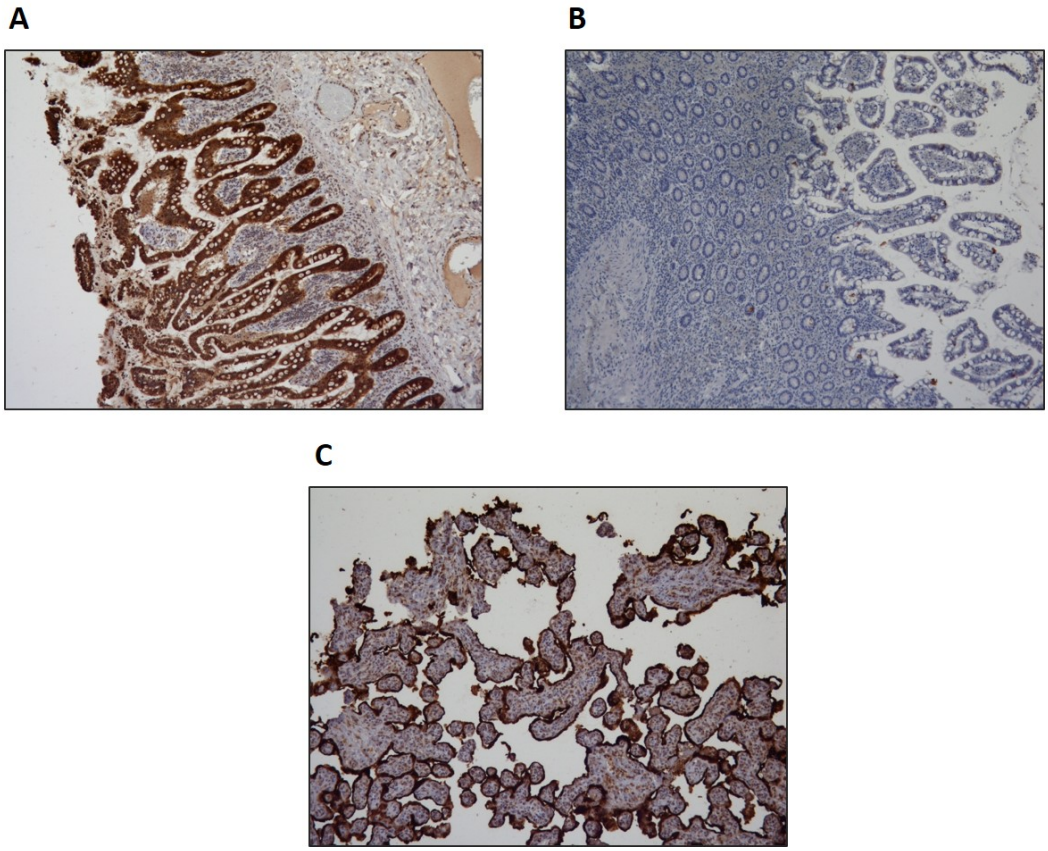

Figure 3.13: **Immunohistochemical staining for 11β-HSD2 in placental explants. (A)** Human bowel (= positive control). **(B)** Human bowel without primary antibody (= negative control). **(C)** Placental explant tissue cultured for five days before it was stained for 11β-HSD2. n=2, representative images are shown. Blue staining = haematoxylin staining, brown staining = 11β-HSD2 staining.

The 11β-HSD2 protein expression after CRH treatment was quantitatively determined by western blot analysis of placental explants. Figure 3.14 B shows the quantification of the western blot (Figure 3.14 A). Again, as with the quantification of mRNA, the same treatment (lane 3 and 4 = 100 nM CRH, lane 5 and 6 = 1 μM CRH in Figure 3.14 A) gave inconsistent responses in the tissue of one placenta suggesting a great intravariability. Moreover, analysis of a second experiment with another placenta (Figure 3.14 C and D) showed in addition to intravariability after treatment with 1 μM CRH an intervariability compared to the first placenta. Intervariability describes the variability between samples collected from different placentas. In these placental explants, the CRH treatment led to a downregulation of 11β-HSD2 protein (Figure 3.14 C and D), whereas in the first placenta this did not happen (Figure 3.14 A).

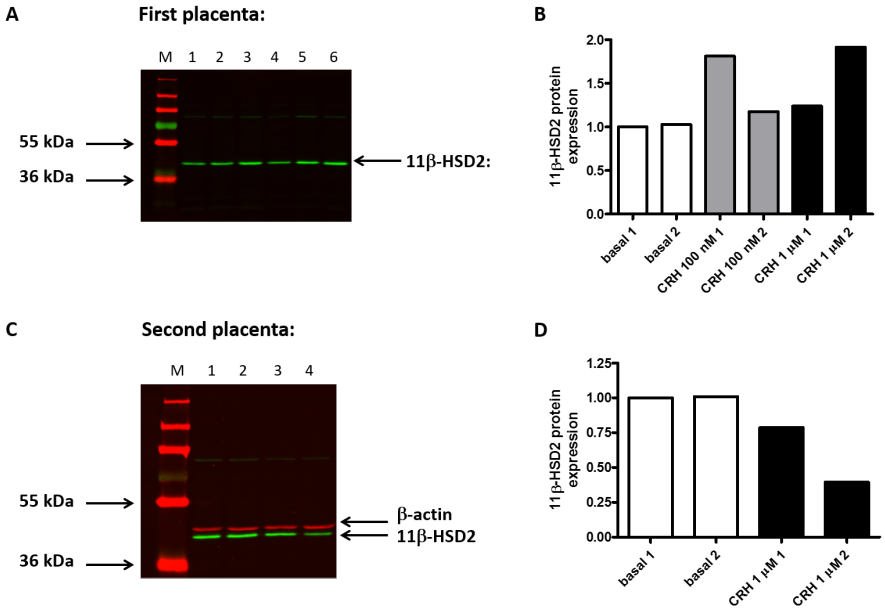

Figure 3.14: **Protein expression of 11β-HSD2 in placental explants.** Placental explants were cultured for five days with 100 nM or 1 μM CRH before protein lysate was prepared and WB for 11β-HSD2 was performed. **(A)** WB of one explant experiment (Lane 1, 2 = basal, lane 3, 4 = 100 nM CRH, lane 5, 6 = 1 μM CRH) with analysis **(B)**, **(C)** WB of another explant experiment (lane 1, 2 = basal, lane 3, 4 = 1 μM CRH) with analysis **(D)**, duplicates for each treatment. Normalisation to β-actin.

3.4.3 Detection of a Glucocorticoid-responsive Gene in Placental Explants

To test glucocorticoid responsiveness of placental explants, expression of FKBP5, a known glucocorticoid-responsive gene [U et al., 2004], was determined. FKBP5 protein was upregulated by 35-fold in placental explants after five days of 1 μM Dex for treatment (Figure 3.15). Comparison of FKBP5 protein amount on day 0 (fresh placenta) with the amount after 5 days of basal culture shows a decline by 25% in FKBP5 protein expression.

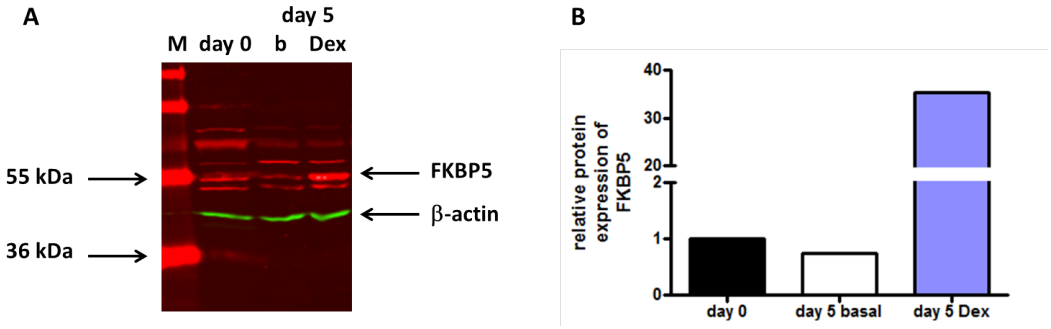

Figure 3.15: **Protein expression of FKBP5 in placental explants.** Placental explants were cultured for five days with 1 μM Dex before protein lysate was prepared and WB for FKBP5 was performed. **(A)** WB of one explant experiment with **(B)** analysis. M=Marker, Lane 1: fresh placenta (day 0), Lane 2: basal day 5, Lane 3: 1 μM Dex day 5. Normalisation to β-actin.

3.5 Discussion of Chapter 3

Placental endocrine function and cell turnover have been shown to be altered in various placenta-associated diseases such as pre-eclampsia and IUGR [Arnholdt et al., 1991; Ishihara et al., 2002; Ruebner et al., 2010]. These alterations can lead to a less efficiently functioning placenta which, as a result, could have detrimental effects on fetal development. In this chapter, I attempted to standardize methodologies that would allow measurement of proliferation, of hCG secretion, and of apoptosis in the placental explant culture. Responsiveness of the system to external stimuli such as CRH and/or LPS was investigated in the placental explant model. Furthermore, an initial characterization of molecules involved in the glucocorticoid metabolism was started in pilot experiments.

Despite many proposed roles of CRH on placental biology, its role, especially in challenging circumstances, has not been fully investigated. It has been suggested that CRH is involved in the onset of labour because it plays a role in maintaining the vascular tone and then late in pregnancy in stimulating secretion of prostaglandins which might be involved in the parturition process [McLean et al., 1995; Clifton et al., 1995; Jones and Challis, 1989; Benedetto et al., 1994]. The concentration of placental CRH rises towards the end of pregnancy and interestingly, in placental diseases the concentration of placental CRH rises even further towards the end of pregnancy than in normal pregnancies [Frim et al., 1988; Laatikainen et al., 1991]. The production of excess CRH might occur due to a possible regulatory function of CRH in disease states. In this study, the capacity of CRH to affect the placental hormone production and placental cell turnover was investigated. The hypothesis of the study was that CRH might play a beneficial role on placental biology and that this effect might only become apparent in case of a challenging environment.

LPS was chosen to activate the receptor TLR4 which has been shown to be expressed in the placenta [Holmlund et al., 2002]. Activation of TLR4 leads to activation of the NFκB pathway and ultimately evokes an inflammatory response by the cell. As the parturition process is characterized by an inflammatory milieu (Figure 1.17), the use of LPS and its provoked inflammatory response mimics features of term and pre-term labour. Also, pregnancy-related diseases such as pre-eclampsia show increased inflammatory processes which might affect the placenta (Chapter 1.3.1.1) and conditions such as obesity and diabetes during pregnancy are characterized by a low-grade inflammation (Chapter 1.3.2).

An interplay of CRH and LPS pathways can be observed in the placenta. For

example, Torricelli et al. [2011] found a higher concentration of CRH mRNA in pre-term placentae with chorioamnionitis than in pre-term placenta without chorioamnionitis. Furthermore, they showed that treatment of placental explants with LPS increased CRH mRNA. Also in a trophoblast cell line model, treatment with LPS led to an upregulated CRH production in JEG-3 cells [Uh et al., 2008]. So, it seems that placental CRH concentration rises possibly through a TLR4-induced mechanism. In addition to CRH treatment, LPS treatment in order to investigate its effect on placental hormone production and cell turnover was carried out. Hypothesis was that LPS might have a detrimental role on placental biology which might be counter-regulated by an elevated CRH concentration.

Regarding detection of trophoblast proliferation, Ki67-staining has been successfully used by other groups to detect proliferation in the placenta and in placental explants [Unek et al., 2014; Matos et al., 2014]. Proliferation of placental explants after CRH and LPS treatment was investigated because some, but not all, groups found that proliferation indices of disease placentae might be altered. Arnholdt et al. [1991] showed that cytotrophoblast proliferation is increased in placentae from pre-eclampsia patients compared to placentae from healthy patients. Jeschke et al. [2006] showed a significantly increased cytotrophoblast proliferation in patients with the HELLP syndrome, but not in patients with pre-eclampsia or IUGR. Smith et al. [1998b] did not find a difference in trophoblast proliferation in placentae from IUGR. Since the cause of the altered trophoblast proliferation is not known yet and since these placental disease phenomenons are associated with elevated CRH levels, it was tested whether CRH (with and without the presence of LPS) is involved in proliferation of trophoblast tissue.

Qualitatively it appears that neither CRH nor LPS treatment had an effect on proliferation of placental explants on day 5 of culture. It might be possible that alterations of proliferation events could have been detected to an earlier time point of the placental explant culture. For example, Arnholdt et al. [1991] and Smith et al. [1998b] showed less proliferation in term than in early gestation. This suggests that investigation of proliferation in placental explants between day 1 and 3 of culture (when the old syncytiotrophobast is lost and the new syncytiotrophoblast is generated) might be a suitable timing for future experiments.

Simán et al. [2001] described the pattern of hCG secretion by placental explants and because the hCG release in our experiments followed this pattern, it was concluded that our experimental protocol of culturing placental explants was

in agreement with the published literature. Treatment with CRH or LPS altered the secretion of hCG between culturing days three and six. Analysis of hCG secretion on day 5 (compared to day 2) revealed that CRH increased hCG production and that LPS showed a possible inhibitory action on hCG secretion. Simultaneous treatment with both LPS and CRH reached a similar level as the basal hCG production, suggesting that the effect of LPS on hCG production was abolished by simultaneous treatment with CRH. Thus, CRH (alone or in co-treatment) seems to have the ability to stimulate hCG production of syncytiotrophoblast. This observation suggests that CRH might play a role in placental diseases as it might maintain hCG secretion which, as described in the introduction (Chapter 1.2.7.2), fulfils many important roles in maintaining pregnancy.

We observed inconsistent responses of placentae which were broadly divided into "'high hCG responders"' and "'low hCG responders"' marked by a significantly different initial hCG release on day one of culture. One problem with this observation is that it is not possible to predict whether a placenta is a high or low hCG responder. There might be clues from the morphological appearance of the placenta during preparation of the placental explants, but this is not an objective criteria. The difference was apparent only after measuring the hCG concentration after the first 24 h of culture. Nevertheless, for the analysis of the experiments in this study, placentae displaying a low hCG production on day one were defined as low hCG responders and placentae displaying a high hCG production on day one were defined as high hCG responders (Figure 3.4). This definition led to interesting differences in the responsiveness of the two groups to stimuli such as CRH and LPS as the low hCG responder placentae showed increased hCG secretion after all treatments suggesting that they might be hyperresponsive to various stimuli.

Comparing these data with data from literature, the relevant literature is characterized by inconsistent findings. Crocker et al. [2004] measured a decreased hCG production of placental explants after treatment with TNF-α. Also, Leisser et al. [2006] demonstrated the inhibitory effect of TNF-α on hCG-β mRNA expression and hCG secretion in primary trophoblast cells isolated from healthy term placentae. Okada et al. [1997] used chorioamnionitis as a disease-related model. They observed less hCG production in tissue fragments cultured for 24 h from pre-term placentae with chorioamnionitis compared to pre-term without chorioamnionitis. Also, they showed that treatment of primary trophoblast cells isolated from healthy placentae with LPS secreted less hCG than control treated cells. These findings are in agreement with my data after LPS treatment of placental explants from high hCG responder placentae. In contrast to these data, some groups postulated a stimula-

tory effect of TNF-α on hCG secretion of trophoblast cells. In cellular models, hCG secretion was increased after TNF-α treatment of JEG-3 and JAR cells [Pedersen et al., 1995; Jiang et al., 2006]. However, the cell line characteristics might have changed the regulation of hormone production and therefore, these data might not be directly comparable to my experiments. Furthermore, Li et al. [1992] showed that TNF-α had a stimulatory effect on hCG production from normal trophoblast cells. On the other hand, this experiment was performed with trophoblasts isolated from first trimester placentae, which might exhibit different characteristics compared to term placentae. But conversely, Leisser et al. [2006] observed a decreased hCG secretion after TNF-α treatment in placental explant culture prepared from first trimester placentae.

Finally, the intra- and intervariability seem to be a common feature when doing experiments with placental explant culture as it has been recognized by Turner et al. [2006]. Because of this and the unpredictability of obtaining a high hCG responder placenta and the difficulty of setting a standard for the characteristic of high hCG responder, it was decided to shift focus towards pursuing experiments with the BeWo cell line (see subsequent chapters 4 to 6).

Apoptosis is a molecular key mechanism that determines the overall responses of placental explants. The changes of hCG secretion by placental explants after different stimuli treatments might be associated with potential alterations in the apoptotic process. Also, apoptotic features are observed during cytotrophoblast differentiation (Chapter 1.2.6.3) linking the two processes of apoptosis and differentiation. It appears that CRH can alter the biochemical differentiation of trophoblasts as it had an effect on the hCG secretion of the placental explants (discussed above). Whether CRH plays a pro- or anti-apoptotic role on placental explant biology is not known. Other studies revealed conflicting results regarding the pro- and anti-apoptotic effect of CRH. Dermitzaki et al. [2002] showed that CRH has a pro-apoptotic effect in pheochromocytoma PC12 cells. An anti-apoptotic effect of CRH in pancreatic cells was demonstrated by Schmid et al. [2011].

Apoptotic processes are also elevated in several placenta-related diseases. For example, apoptosis is increased in placentae from pre-eclamptic and IUGR pregnancies [Leung et al., 2001; Ishihara et al., 2002]. Saglam et al. [2013] showed more apoptosis in the fetal membranes in preterm premature rupture of fetal membranes (PPROM). And Levy et al. [2002] showed a higher rate of trophoblast apoptosis in fetal growth restriction accompanied by an elevated p53 expression. These examples demonstrate a deregulation of trophoblast apoptosis during several different

placental disease states. In my study, co-treatment with LPS was performed to investigate whether CRH has effects on trophoblast apoptosis under conditions which are characterized by activation of TLR4.

The M30-staining was used as a qualitative measurement of apoptosis. Again, as it was shown for hCG secretion, placental explants showed inconsistent and variable results regarding apoptosis. In order to quantify apoptotic events in placental explants, multiplex ELISA for four apoptotic marker molecules (cleaved caspase-3, cleaved PARP, p53, phospho-p53) was performed. The staurosporine treatment in the initial experiment (shown in the Material and Methods Chapter) revealed that the sample protein concentration in the subsequent experiments with CRH and LPS was probably too low in order to detect differences in p53 and pp53 expression. However, the experiments were pursued in order to detect potential differences in the cleavage of caspase-3 and PARP.

The culture conditions during the placental explant culture seem to have an effect on the activation of caspase-3 as after four and five days of culture more activated caspase-3 was detected. However, none of the other markers was changed. This might be explained by the fact that the concentration of protein was not enough for detecting changes (as explained in the paragraph above). On the other hand, it could also mean that even though caspase-3 is activated, the apoptotic process is executed incompletely suggesting that the tissue is not fully apoptotic.

Treatments with CRH and LPS did not lead to the detection of differences in the expression of cleaved caspase-3, cleaved PARP, p53 and pp53. Possibly, CRH and LPS do not affect apoptosis in placental cells. Or as the same experiment was performed with BeWo cells by another student of our group and only a minimal effect of LPS and CRH treatment on the expression of cleaved caspase-3 was detectable, this shows that this method might not be the most suitable for tissues as it is always more difficult to detect differences in a heterogenous tissue than in a homogenous cell line.

Taken together, in this study due to limitations of detection methods and the intervariable nature of placental explants, no reproducible effect of CRH and LPS on placental explant apoptosis could be detected.

The initial characterization of molecules involved in glucocorticoid action in placental tissue showed that 11β-HSD2, P-gp, and FKBP5 are expressed in placental explants. In my study, 11β-HSD2 and P-gp mRNA were upregulated after treatment with dexamethasone. In a similar manner, Audette et al. [2010] observed an upregulation of 11β-HSD2 mRNA after treating placental explants with dexam-

ethasone. However, quantification of mRNA from the placental explants showed a great variability between responses with the same treatment in my study. The quality of the isolated mRNA was not good (low RNA yield, contamination of RNA was detected by a low A260/280 and A260/230 ratios) and this might be a reason for the variability.

Also the quantification of protein isolated from the placental explants showed intravariability. Moreover, treatment with CRH had an inconsistent effect on 11β-HSD2 protein expression suggesting an intervariability between placentae. The problems could arise from the different percentages of syncytiotrophoblast, cytotrophoblast, mesenchymal cells and other cell types in the placental fragments. Also possibly, the different explant samples could vary in their CRH receptor expression as well as in CRH receptor linkage to G proteins.

Taken together, the preliminary data shows that placental explants retain the trophoblast expression pattern of 11β-HSD2 as most of the protein is expressed in the syncytiotrophblast layer. Furthermore, 11β-HSD2 is inducible in placental explants upon stimulation as shown by treatment with dexamethasone. Also the induction of FKBP5 (a known glucocorticoid responsive gene) by dexamethasone demonstrates the responsiveness of placental tissue to glucocorticoids. However, quantification of mRNA and protein isolated from placental explants revealed technical problems which were another reason to pause performing experiments on placental explants and to focus most subsequent studies on the BeWo cell line.

As a consequence of the various difficulties (intra- and intervariability of placental explants in hormone secretion and protein expression, poor mRNA quality, high cost of performing placental explant experiments), the cell line BeWo was used as an alternative placental model system (see chapter 4 for characterization of BeWo cell line) in the subsequent chapters. Nevertheless under appropriate standardization of methodologies, placental explants can allow a suitable system to investigate placental endocrine function as well as glucocorticoid-driven mechanisms.

Chapter 4

Characterization of BeWo Cells as a suitable Model System

The placental explant culture experiments (shown in the previous chapter 3) identified a series of important issues in the investigation of placental responses to stress hormones. This led to seek alternative models to increase robustness of experiments. I chose the human choriocarcinoma cell line BeWo, a well-established model for placental differentiation, as an *in vitro* cell model for the studies investigating the trophoblast enzyme 11β-HSD2 and the trophoblast glucocorticoid metabolism (Chapter 5 and 6).

I established in the initial stages of my investigation the characteristics of BeWo cells regarding their cell turnover, endocrine capacity and expression of proteins involved in the stress response and glucocorticoid action molecular machinery such as CRH and its receptors, gluco- and mineralocorticoid receptor, and the enzyme 11β-HSD2.

In contrast to primary trophoblast cells, BeWo cells require treatment with a cAMP-stimulator such as forskolin to initiate the differentiation process [Wice et al., 1990; Delidaki et al., 2011]. The differentiation process involves cell fusion and upregulation of key proteins including hormones such as hCG and progesterone (Chapter 4.1). In addition to cell fusion and endocrine capacity, expression of molecular components which are involved in the cellular stress response and glucocorticoid action were characterized (Chapter 4.2).

4.1 Differentiation of BeWo Cells with Forskolin

4.1.1 Fusion of BeWo Cells

Cell fusion was investigated with a nuclear staining, immunodetection of the cell adhesion molecule E-Cadherin (= E-Cadherin staining), and a fluorescent cell-labeling assay which monitors fusion processes (see chapter 2.5.1 for cell-labeling assay details). The brightfield images with nuclei staining showed areas of condensed nuclei after 48 h of forskolin treatment ("'forskolin 24 h + basal 24 h"' and "'forskolin 48 h"') (Figure 4.1) suggesting presence of fused BeWo cells. The E-Cadherin staining showed an extensive reorganization and remodelling of the E-Cadherin distribution after 48 h of forskolin treatment ("'forskolin 24 h + basal 24 h"' and "'forskolin 48 h"') (Figure 4.2) compared to basal conditions also suggesting that BeWo cells have fused. The fluorescent fusion assay showed large yellow stained areas which represent the fused cells after 48 h of forskolin treatment (Day 4) (Figure 4.3). These detection methods also showed after 24 h of forskolin treatment only few areas with condensed nuclei (nuclear staining) and break-up of the E-Cadherin structure only in some areas suggesting that the fusions process has started after 24 h of forskolin treatment, but was not sufficient for complete fusion of BeWo cells.

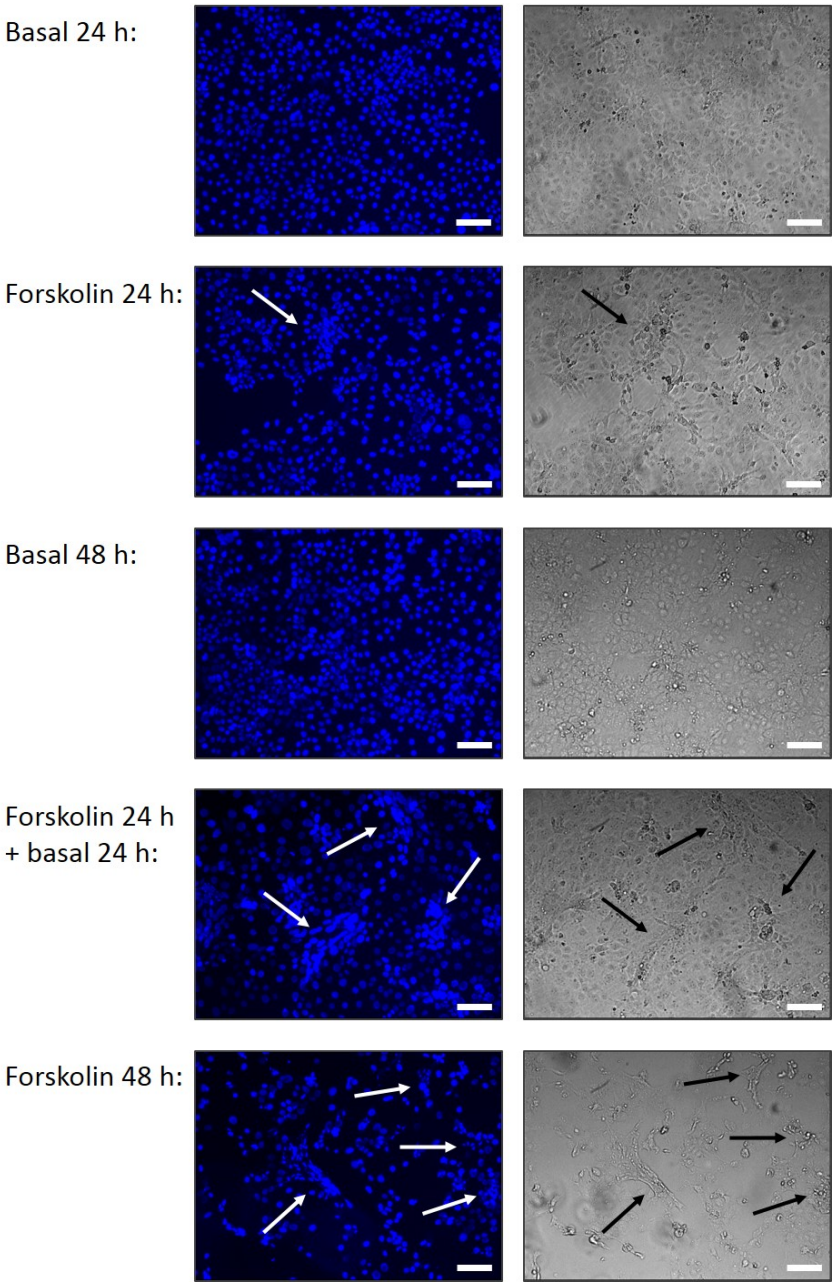

Figure 4.1: **Fusion of BeWo cells demonstrated by brightfield microscopy (right) with DAPI staining (left).** BeWo cells were cultured with and without 100 μM forskolin for various time points before their nuclei were stained with DAPI, which is shown in blue. Representative images are shown, n=3, bar = 100 μm, arrows indicate areas with fused cells.

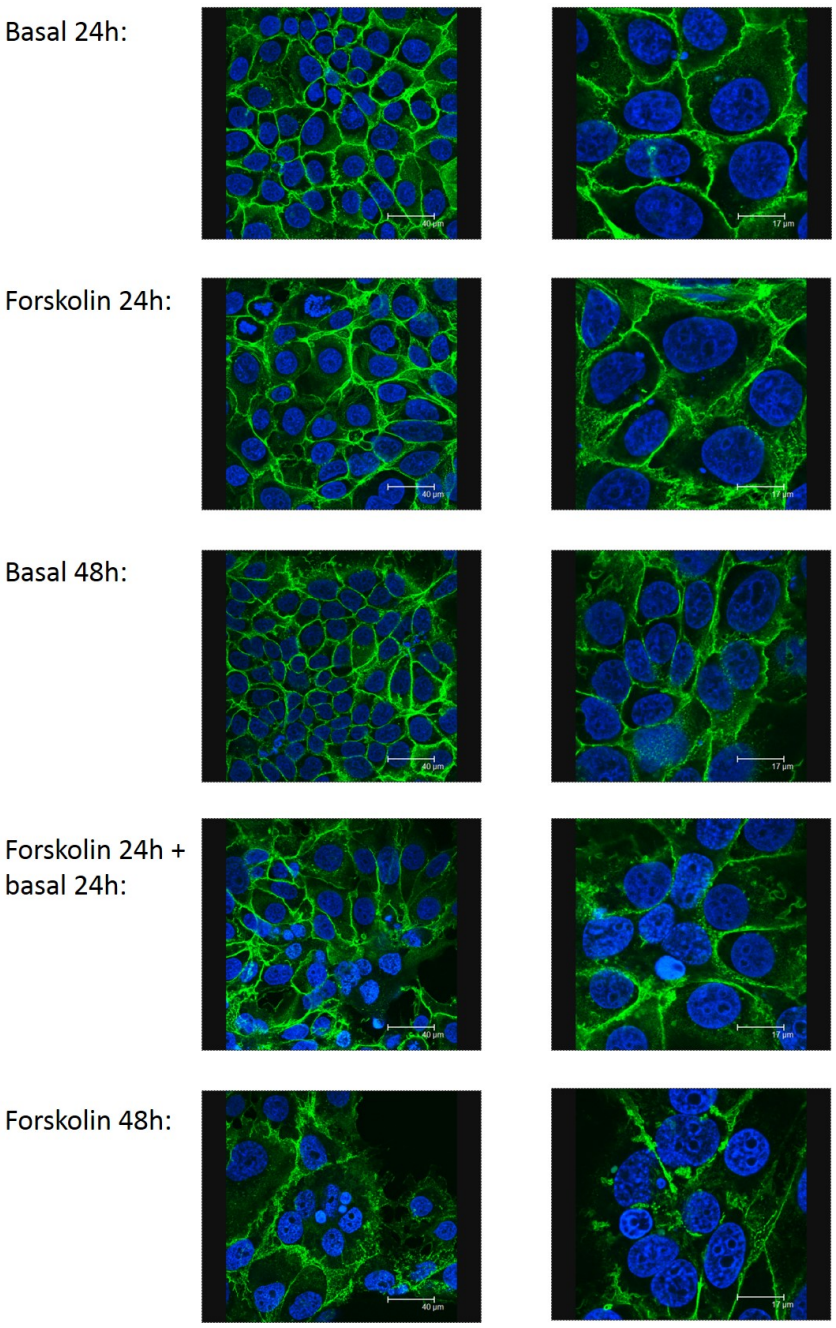

Figure 4.2: **Fusion of BeWo cells demonstrated by E-Cadherin staining.** BeWo cells were cultured with and without 100 μM forskolin for various time points before they were stained for E-Cadherin (a membrane protein), which is shown in green. Nuclei are stained blue with DAPI. Representative images are shown, n=3, Left: bar = 40 μm, right: bar = 17 μm.

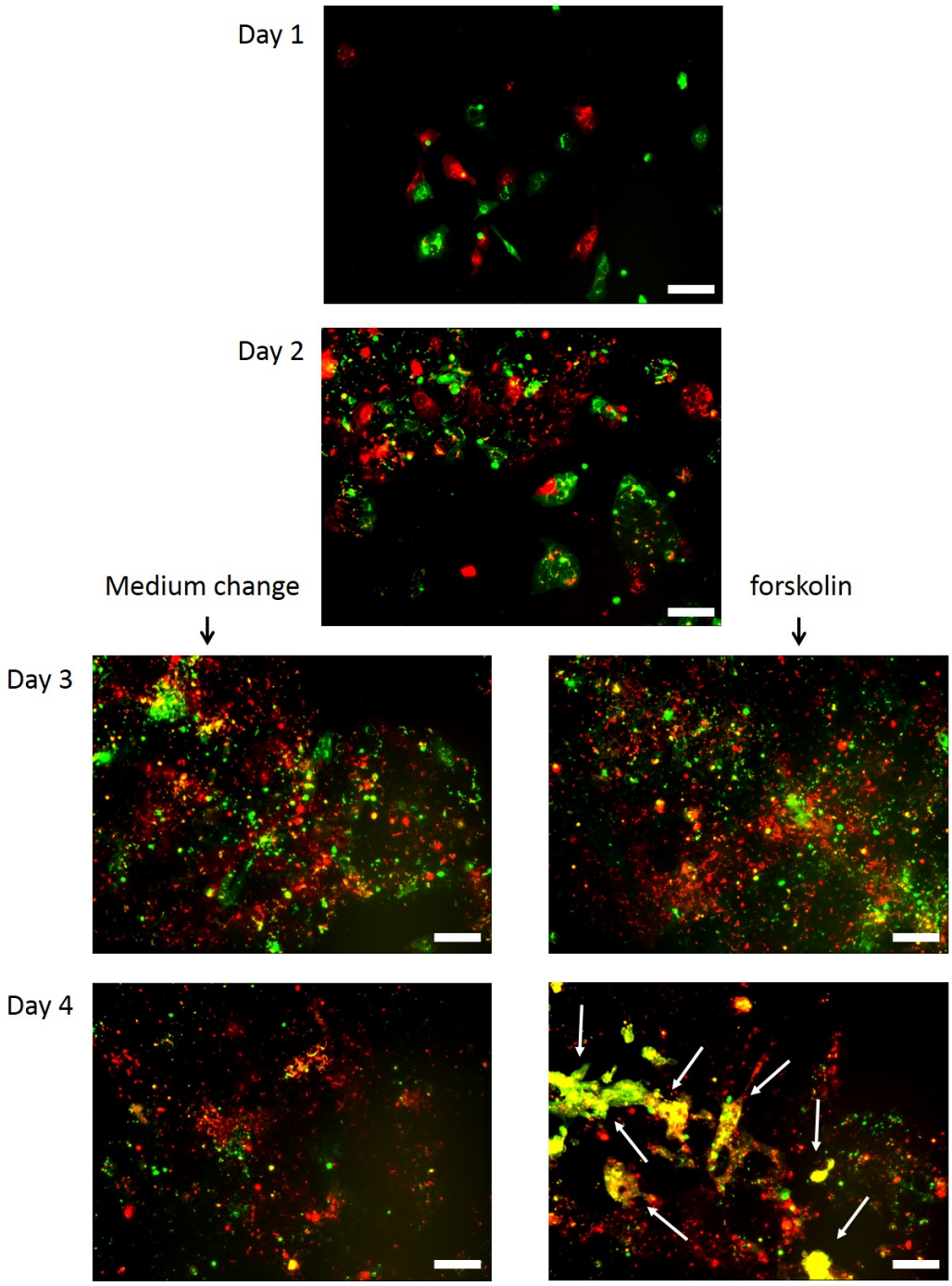

Figure 4.3: **Fusion of BeWo cells demonstrated by monitoring two BeWo cell populations labelled with two different cell tracer dyes (Dil or DiO), seeded together.** BeWo cells were grown for two days before treating them with 100 μM forskolin for 24 (= Day 3) and 48 h (= Day 4). Green = DiO staining, red = Dil staining, yellow = Dil and DiO staining. Representative images are shown, n=2, bar = 100 μm, arrows indicate fused cells.

4.1.2 Expression of Fusogenic Genes and their Receptors

Successful differentiation and cell fusion of trophoblast cells depend on upregulation of expression of the endogenous fusogenic retroviral proteins Syncytin-1 and Syncytin-2 which bind to the receptors ASCT2 and MFSD2, respectively. In agreement with the established models of trophoblast differentiation, treatment of cells with forskolin showed qualitatively the protein expression of Syncytin-1 in the BeWo cell line (Figure 4.4 A). Both Syncytin-1 and -2 were significantly increased by 2.1-fold and 8.2-fold, respectively, after 24 h of 100 μM forskolin exposure at mRNA level compared to control-treated cells (Figure 4.4 B, C). After 48 h of 100 μM forskolin stimulation, there was a reduction of the forskolin-induced Syncytin-1 and -2 mRNA expression compared to the 24 h time point, although their expression was still significantly upregulated by 1.6-fold and 1.7-fold, respectively, compared to control-treated cells (Figure 4.4 D, E).

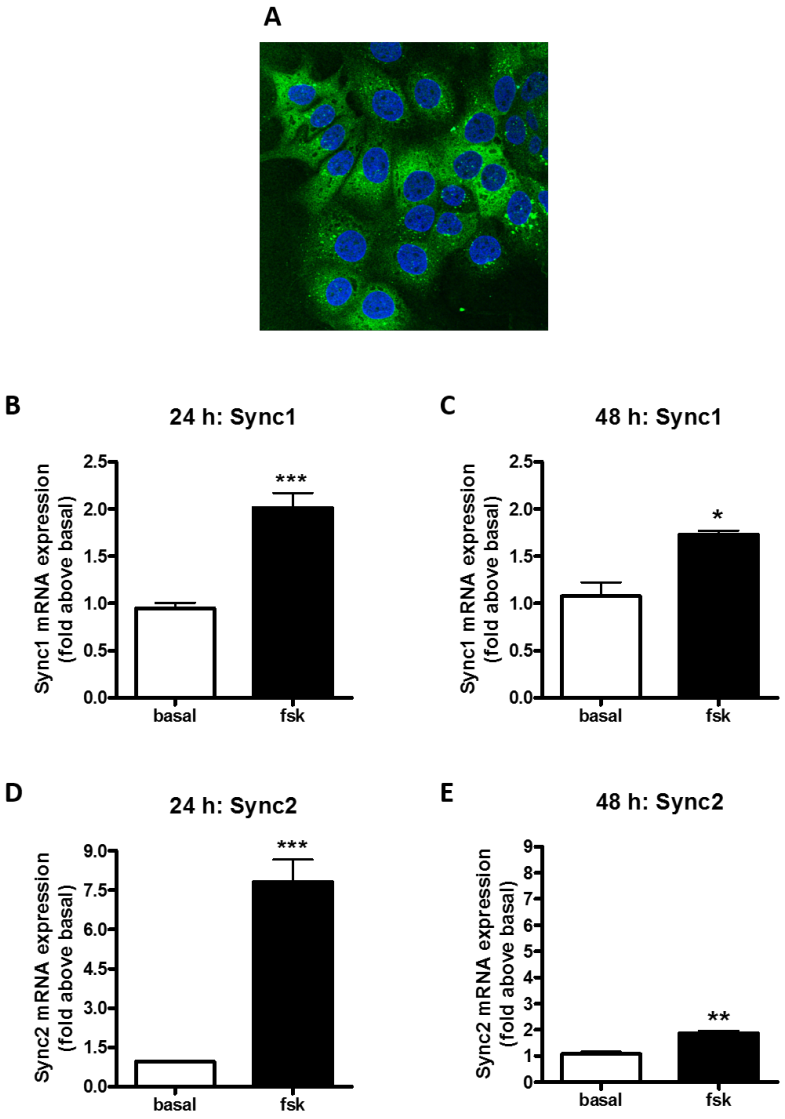

Figure 4.4: **Expression of Syncytin-1 and -2 after forskolin treatment.** BeWo cells were treated with 100 μM forskolin for 24 h before **(A)** staining for Syncytin-1 protein was performed or for 24 h and 48 h before **(B-E)** mRNA was extracted and qRT-PCR for Syncytin-1 and -2 was performed. **(A)** Green = Syncytin-1, blue (DAPI staining)= nuclei. Representative image is shown. **(B-E)** Normalization to 18S rRNA, n=6 (24 h) and n=3 (48 h), data are expressed as mean values ± SEM, t-test: $p<0.05$ (=*), $p<0.01$ (=**), $p<0.001$ (=***).

I also investigated the expression of the Syncytin-1 receptor ASCT2 mRNA which showed a trend of downregulation after forskolin treatment, despite failure to reach statistical significance (Figure 4.5 A), whereas the Syncytin-2 receptor MFSD2 mRNA was significantly up-regulated after 24 h of 100 μM forskolin treatment (Figure 4.5 B).

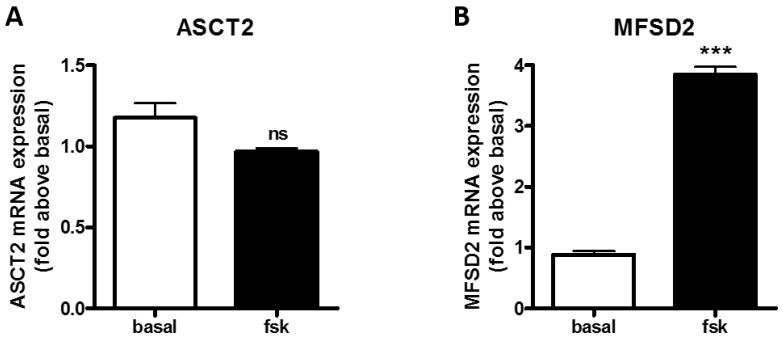

Figure 4.5: **Expression of the Syncytin-1 and -2 receptors (ASCT2 and MFSD2) after forskolin treatment.** BeWo cells were treated with 100 μM forskolin for 24 h before mRNA was extracted and qRT-PCR for **(A)** ASCT2 and **(B)** MFSD2 was performed. qRT-PCRs were normalized to RPLP0 mRNA expression, n=3, data are expressed as mean values ± SEM, t-test: ns = non-significant, $p<0.001$ (=***).

4.1.3 Caspase-3/7 Activation during the Fusion Process

The effect of forskolin on BeWo cell apoptosis was investigated because activation of the trophoblast fusion process involves apoptotic mechanisms (Chapter 1.2.6.3).

Treatment of BeWo cells with 100 μM forskolin caused a time-dependent increase in caspase-3/7 activation (Figure 4.6). Non-treated cells (Figure 4.6 A) displayed only a few scattered cells positively stained with a specific antibody for actived caspase-3/7. After 24 h treatment with forskolin, there was an increase in the number of signal-positive cells mostly found within areas of cell fusion into a syncytium (Figure 4.6 B). In contrast, non-fused areas did not exhibit a staining for activated caspase-3/7. After 48 h of exposure to forskolin, large areas of the cells were fused and were positive for activated caspase-3/7 staining (Figure 4.6 C).

Interestingly, the medium change after 24 h also had an influence on the activation of caspase-3/7 in the cells. The non-treated cells without medium change had more green-stained cells than the non-treated cells with medium change (Figure 4.6 compare D with B). This effect was also observed in forskolin-treated cells as cells treated with forskolin for 48 h without a medium change displayed more signal-positive cells than cells treated with forskolin for 48 h with a medium change (Figure 4.6 compare E with C).

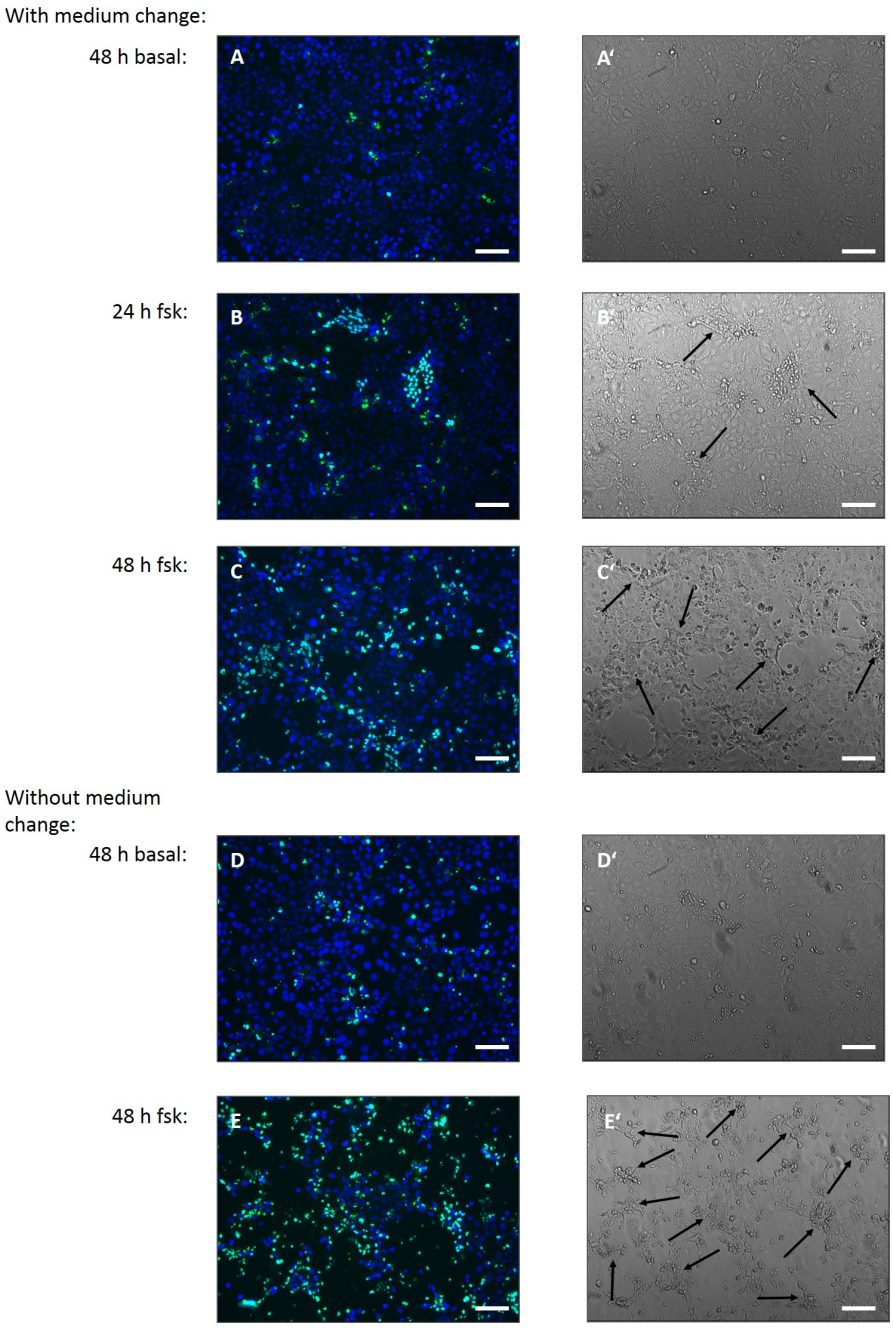

Figure 4.6: **Activated caspase-3/7 after 24 h and 48 h of forskolin treatment (with and without medium change after 24 h)**. BeWo cells were cultured with and without 100 μM forskolin for 24 h and 48 h before a staining for activated caspase-3/7 was performed. Nuclei are stained blue with DAPI. Left: overlay of DAPI and caspase-3/7 staining (green), right: brightfield image. Representative images are shown, n=2, bar = 100 μm, arrows in the brightfield images indicate areas of fused cells.

To obtain quantitative information, I employed an additional approach based on the detection of a fluorescent substrate which is cleaved by activated caspase-3 and -7 (= ApoONE assay, see chapter 2.7.3 for details). This quantitative measurement of activated caspase-3/7 after 24 h of 100 μM forskolin treatment showed a significant increase in activated caspase-3/7 by 1.8-fold (Figure 4.7).

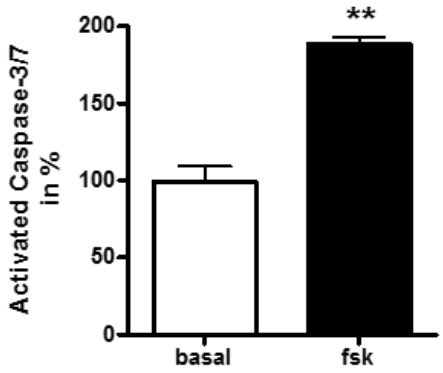

Figure 4.7: **Activated caspase-3/7 after 24 h of forskolin treatment in BeWo cells.** BeWo cells were cultured with and without 100 μM forskolin for 24 h before the ApoONE assay was performed which measures activated caspase-3/7. Data are expressed as mean values ± SEM, n=3, t-test: $p<0.01$ (=**).

4.1.4 Hormone Production of BeWo Cells

Having established that upon forskolin stimulation the BeWo cells fuse, the fusogenic machinery is upregulated and proapoptotic cascades are activated, I then investigated the BeWo cell capacity to secrete hormones. The placenta is an endocrine organ secreting peptide and steroid hormones such as hCG, progesterone (P4), and estradiol (E2). The capacity of BeWo cells for the production of hCG, P4, E2 and cortisol was investigated in un- and differentiated BeWo cells. It has been established that placental cell lines have the capacity to synthesize and release steroid and peptide hormones, especially under conditions of induced differentiation [Knoth et al., 1969; Pattillo et al., 1971; Sullivan, 2004].

4.1.4.1 hCG, P4, and E2 Production of BeWo Cells

Human CG, P4, and E2 secretion was monitored at various time points (24 and 48 h) after 100 μM forskolin treatment. Figure 4.8 A shows a significant 8-fold increase of hCG release after 24 h of forskolin treatment (mean 3043 mIU/L ± 160 SEM) compared to non-treated cells (mean 374 mIU/L ± 11 SEM). The hCG production was further increased after 48 h of forskolin stimulation, although basal hCG release was also slightly increased (mean concentration of 5383 mIU/L ± 96 SEM and 612 mIU/L ± 62 SEM, respectively).

Figure 4.8 B shows that forskolin treatment led to a significant increase in P4 production compared to control-treated cells by 3-fold both after 24 h and 48 h.

In contrast, forskolin treatment for 24 and 48 h did not affect estradiol secretion compared to control-treated cells (Figure 4.8 C).

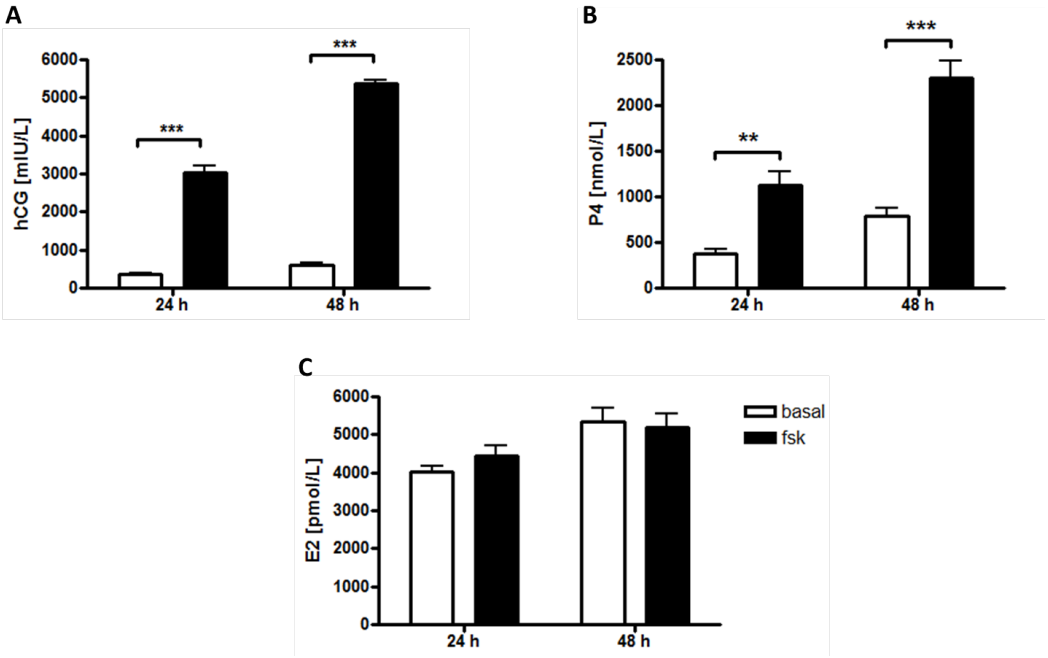

Figure 4.8: **hCG, P4, and E2 production of BeWo cells.** BeWo cells were cultured with and without 100 μM forskolin for 24 h and 48 h and the concentration of secreted hCG, P4, and E2 was measured in the supernatant. Hormone concentration was normalized to protein concentration of BeWo cells. Data are expressed as mean values ± SEM, n=4, t-test: $p<0.01$ (=**), $p<0.001$ (=***).

E2 Production is dependent on FBS

The enzyme cytochrome P450 17A1 (= steroid 17-alpha-hydroxylase), which is encoded by the CYP17A1 gene, is lacking in the placenta and as a consequence the placenta can not produce estrogen *de novo*. *In vivo*, the placenta is supplied with DHEA and DHEAS as precursor molecules for estrogens (see "'Estrogen"' in chapter 1.2.7.2) and the enzymes sulfatase, 3β-HSD, 17β-HSD, and P450 aromatase are involved in the production of the hormones estrone and estradiol (E2). Similar to the *in vivo* situation, BeWo cells require precursor molecules for E2 production. Ugele and Simon [1999] showed that BeWo cells can uptake DHEA-S. Also it has been shown by Pattillo et al. [1972] that BeWo cells can produce E2 from DHEA. Furthermore, Bahn et al. [1981] saw that BeWo cells can produce E2 when FCS is available in the cell culture.

Since in our BeWo cell culture we could measure E2 secretion, I investigated whether E2 production was dependent on precursors found in cell culture medium. BeWo cells produced estradiol in an FBS concentration-dependent manner as shown in Figure 4.9 leading to the conclusion that fetal bovine serum contains the precursor molecules.

4.1.4.2 Cortisol Production of BeWo Cells

Because of the lack of the enzyme steroid 21-hydroxylase, which is encoded by CYP21A2, cortisol can not be produced by the placenta *de novo* [Sullivan, 2004]. *In vitro*, BeWo cells in their undifferentiated state (without forskolin treatment) did not produce any detectable levels of cortisol (mean cortisol concentration = 5.33 nmol/L ± 0.091 SEM) as the F12K medium with 10% FBS supplement contained 5.62 nmol/L (± SEM) itself (data not shown). Treatment with 100 μM forskolin led to a small (24%), but significant, increase of cortisol in the supernatant from 5.33 nmol/L (± 0.091 SEM) in basal condition to 6.60 nmol/L (± 0.287 SEM) after 24 h and from 6.84 nmol/L (± 0.790 SEM) in basal conditions to 10.18 nmol/L (± 1.079 SEM) (=49%) after 48 h (Figure 4.10) suggesting that the immortalized BeWo cell line has acquired some ability to produce small amounts of cortisol.

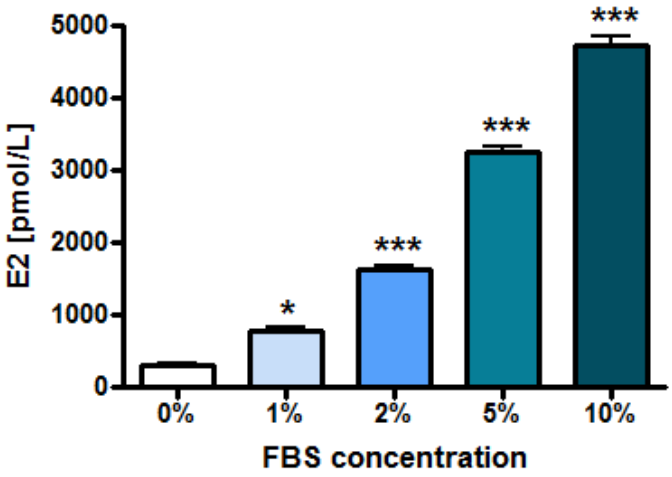

Figure 4.9: **FBS-dependent E2 production of BeWo cells.** BeWo cells were cultured with different concentrations of FBS (0-10%) for 24 h and the concentration of secreted E2 was measured in the supernatant. E2 concentration is normalized to protein concentration of BeWo cells. Data are expressed as mean values ± SEM, n=3, 1-way ANOVA, Bonferroni Posttest: $p<0.05$ (=*), $p<0.001$ (=***), compared to 0% FBS.

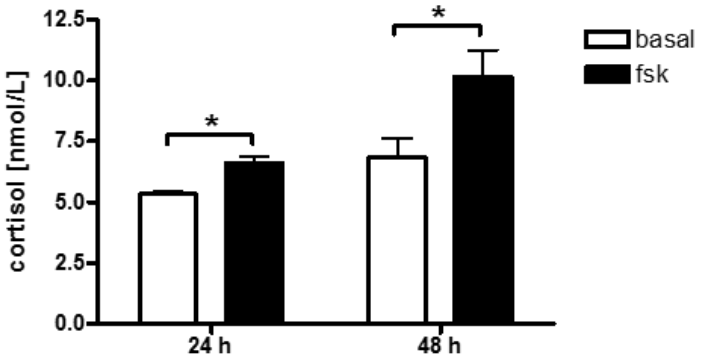

Figure 4.10: **Cortisol production of BeWo cells.** BeWo cells were cultured with and without 100 μM forskolin for 24 h and 48 h and the concentration of cortisol was measured in the supernatant. Cortisol concentration is normalized to protein concentration of BeWo cells. Data are expressed as mean values ± SEM, n=4, t-test: $p<0.05$ (=*).

4.2 Expression of Molecules involved in Stress Response

CRH, the master regulator of the stress axis (HPA axis), is also expressed in the placenta (Chapter 1.1.2.2) playing a potential role in regulatory processes during trophoblast development and feto-maternal communication. Also, the presence of the glucocorticoid barrier in the placenta plays a key role for the appropriate development of the fetus. The main component of the glucocorticoid barrier is the enzyme 11β-HSD2 which converts cortisol into its inactive form cortisone (Chapter 1.1.5). Additionally, transporter molecules such as P-gp which can transport cortisol out of the cell are components of the glucocorticoid barrier (Chapter 1.1.6). Furthermore, the expression of receptors which bind cortisol, namely the gluco- and mineralocorticoid receptor (Chapter 1.1.3), determines the action of cortisol within the cells. In this chapter, to obtain a better understanding of the molecules involved in the adaptive mechanisms of stress responses, I investigated the expression of hormones and receptors in BeWo cells which are involved in the stress response and glucocorticoid action.

In vivo, the placenta expresses the hormone CRH and its two receptors CRH-R1 and -R2, the two steroid receptors glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), the enzyme 11β-HSD2, and the transporter molecules P-gp and BCRP and so it was important to characterize the expression of these molecules in the BeWo cell line. Furthermore, the expression of these molecules after forskolin-induced differentiation was investigated.

4.2.1 Expression of CRH, CRH-R1 and -R2 in BeWo Cells

CRH might coordinate placental glucocorticoid action because a positive-forward-loop between CRH and cortisol in the placenta has been demonstrated (Chapter 1.1.2.2, Robinson et al. [1988]). In the BeWo cells, mRNA expression of CRH and both of its receptors CRH-R1 and CRH-R2 was significantly upregulated by 2.2-, 3.0- and 2.1-fold, respectively, after 24 h of 100 μM forskolin treatment compared to control-treated cells (Figure 4.11). In case mRNA is translated into protein, this suggests that differentiated BeWo cells are more responsive to CRH cellular action.

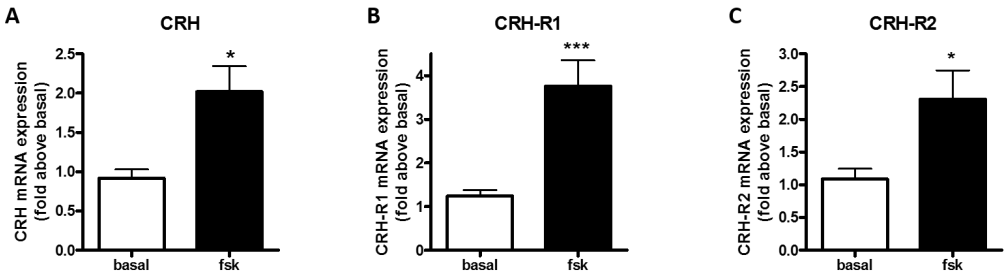

Figure 4.11: **mRNA expression of CRH, CRH-R1, and CRH-R2 in BeWo cells after forskolin treatment.** BeWo cells were treated with 100 μM forskolin for 24 h before mRNA was isolated and qRT-PCR for CRH **(A)**, CRH-R1 **(B)**, and CRH-R2 **(C)** was performed. Samples were normalized to GAPDH mRNA expression, data are expressed as mean values ± SEM, n=6, t-test: $p<0.05$ (=*), $p<0.001$ (=***).

A qualitative immunostaining for CRH-R1 and -R2 revealed their protein expression in forskolin-treated BeWo cells (Figure 4.12).

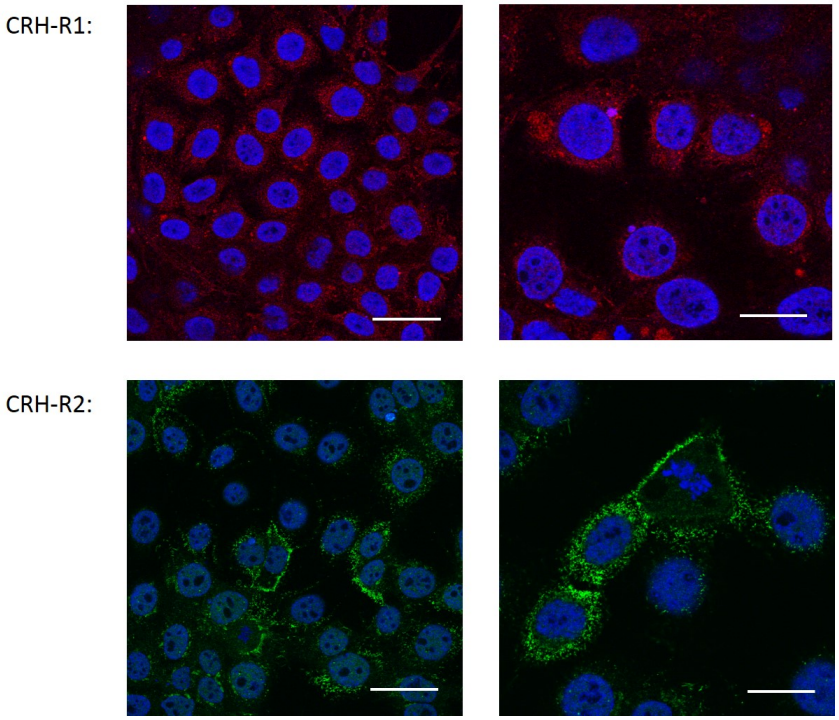

Figure 4.12: **CRH-R1 and -R2 staining in BeWo cells after forskolin treatment.** BeWo cells were treated with 100 μM fsk for 24 h before an immunostaining for CRH-R1 and -R2 was performed. Only images of forskolin-treated cells are shown. Secondary antibody for CRH-R1 staining was ALEXA-633, shown in red. Secondary antibody for CRH-R2 was ALEXA-488, shown in green. Blue DAPI-staining of the nuclei. Representative images are shown, left images: bar = 47.62 μm, right images: bar = 24.83 μm.

4.2.2 Expression of GR and MR in BeWo Cells

Previous studies identified MR expression in the placenta throughout pregnancy in the cytotrophoblast cells as well as in the syncytiotrophoblast [Hirasawa et al., 2000]. The GR was also identified in cytotrophoblast cells and to a lesser degree in syncytiotrophoblast [Lee et al., 2005]. Using qRT-PCR, the steroid receptor GR and MR mRNA were identified in the BeWo cells (Figure 4.13). GR mRNA expression was not affected by 100 μM forskolin treatment (Figure 4.13 A), whereas MR mRNA expression was significantly downregulated by 72% and 34%, respectively, after 24 h and 48 h of forskolin treatment (Figure 4.13 B). Analysis of GR and MR protein expression was not successful because of problems with antibodies (see chapter 2.4.6.3 for details).

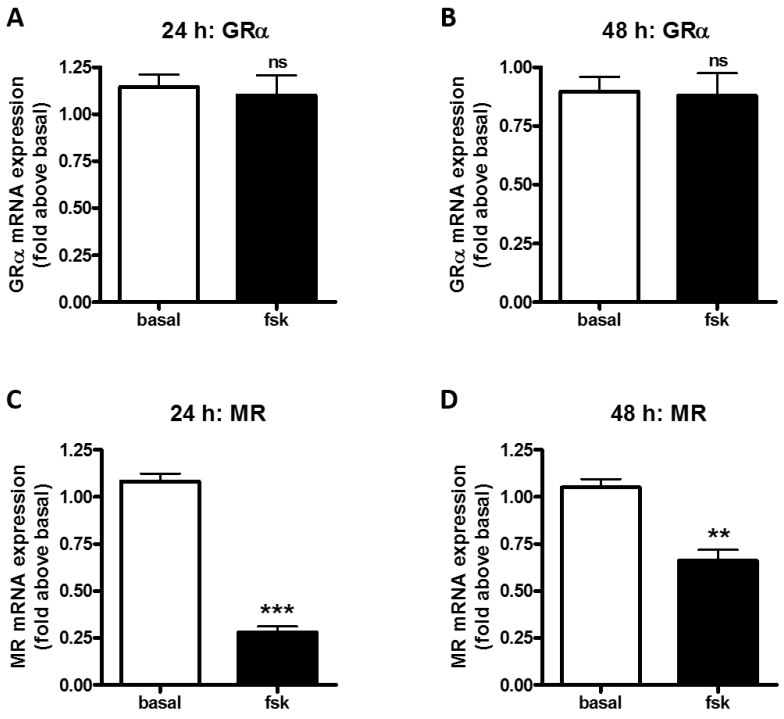

Figure 4.13: **mRNA expression of GRα and MR in BeWo cells after forskolin treatment.** BeWo cells were treated with 100 μM forskolin for 24 h (left graphs) or plus another 24 h of fresh medium (= 48 h, right graphs) before mRNA was isolated and qRT-PCR for GRα **(A)** and MR **(B)** was performed. Samples were normalized to 18S rRNA expression, n=6 (for 24 h), n=3 (for 48 h), data are expressed as mean values ± SEM, t-test: ns = non-significant, $p<0.01$ (=**), $p<0.001$ (=***).

4.2.3 Expression of 11β-HSD2 in BeWo cells

Because the enzyme 11β-HSD2 inactivates cortisol, its expression is extremely important in the regulation of glucocorticoid action within cells. In the placenta, 11β-HSD2 is highly expressed in the syncytiotrophoblast layer as it was shown previously (Figure 3.13 in Chapter 3.4.2). Figure 4.14 A shows that mRNA expression of 11β-HSD2 was highly upregulated by 30-fold by forskolin treatment after 24 h. However, this increase was transient and prolonged stimulation with forskolin for 48 h led to an increase by only 4.8-fold (Figure 4.14 B).

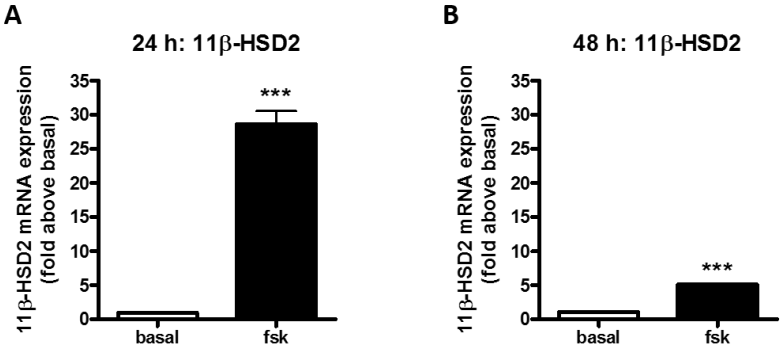

Figure 4.14: **mRNA expression of 11β-HSD2 in BeWo cells after forskolin treatment.** BeWo cells were treated with 100 μM forskolin for **(A)** 24 and **(B)** 48 h before mRNA was isolated and a qRT-PCR for 11β-HSD2 was performed. qRT-PCR was normalized to 18S rRNA expression, n=6 (for 24 h), n=3 (for 48 h), t-test: $p<0.001$ (=***).

Immunostaining for 11β-HSD2 in BeWo cells demonstrated cytoplasmic expression (Figure 4.15). Treatment with forskolin for 24 h led to a substantial increase in 11β-HSD2 immunostaining compared to basal condition.

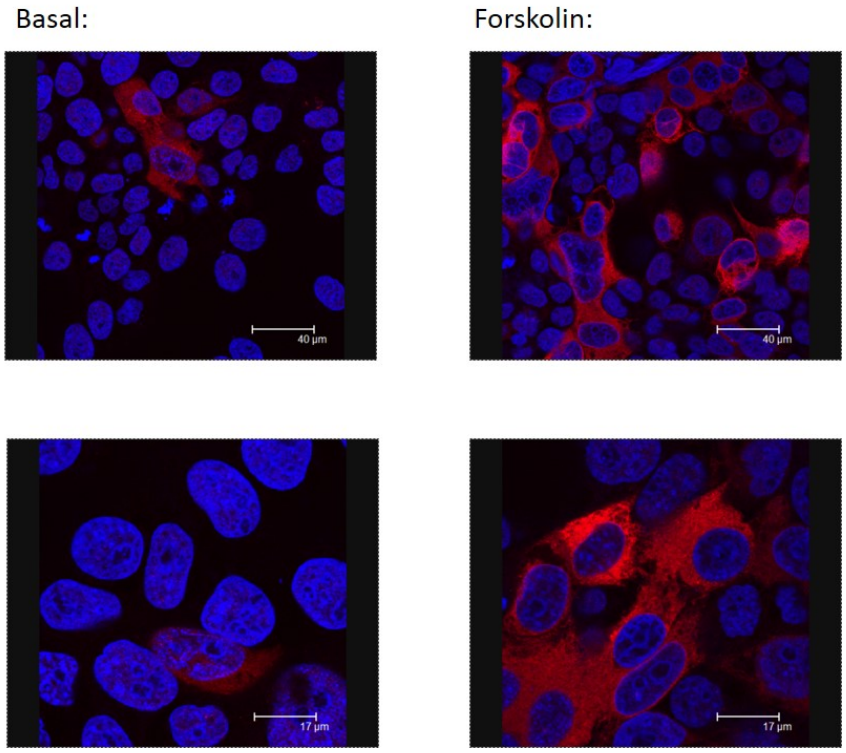

Figure 4.15: **11β-HSD2 staining in BeWo cells after forskolin treatment.** BeWo cells were treated with 100 μM forskolin for 24 h before an immunostaining for 11β-HSD2 was performed. Secondary antibody used was ALEXA-633, shown in red. Blue DAPI-staining of the nuclei. Representative images are shown, n=3, top: bar = 40 μm, bottom: bar = 17 μm.

Cortisol Inactivation in BeWo Cells

As the enzyme 11β-HSD2 inactivates cortisol, the measurement of its activity in addition to the mRNA and protein expression was very important. Furthermore, since forskolin treatment led to a very high increase in 11β-HSD2 expression, it was hypothesized that forskolin-treated differentiated BeWo cells would be able to metabolize cortisol much more effectively than undifferentiated BeWo cells. In order to test this hypothesis, an 11β-HSD2 activity assay which indirectly measures 11β-HSD2 activity was designed.

BeWo cells were treated with different concentrations of exogenous cortisol in the presence or absence of forskolin treatment for 24 h. At the end of the incubation period remaining cortisol was measured in the supernatant. The BeWo cells produced only very low amounts of cortisol (1.27 nmol/L) after forskolin treatment for 24 h (Chapter 4.1.4.2) which was negligible for this method.

Figure 4.16 A and B show that exogenous cortisol was metabolized differentially by un- and differentiated BeWo cells (with and without forskolin treatment). In undifferentiated BeWo cells (Figure 4.16 A), 85% of the 100 nM exogenous cortisol was metabolized, 65% of the 250 nM cortisol, and around 35% of the 500, 1000, 1500 and 2000 nM cortisol. In differentiated BeWo cells (Figure 4.16 B), 100, 250, 500 nM of exogenous cortisol were metabolized in excess of 95%. Treatment with 1000, 1500 and 2000 nM of cortisol led to its inactivation by 83%, 70% and by 56%. This result suggests that forskolin treatment leads to a more effective inactivation of cortisol presumably by increased expression of 11β-HSD2 which is in agreement with the forskolin-induced upregulation of 11β-HSD2 expression (Chapter 4.2.3).

This assay successfully monitored the activity of the enzyme 11β-HSD2 in an indirect manner and was used in the subsequent experiments to determine the 11β-HSD2 activity.

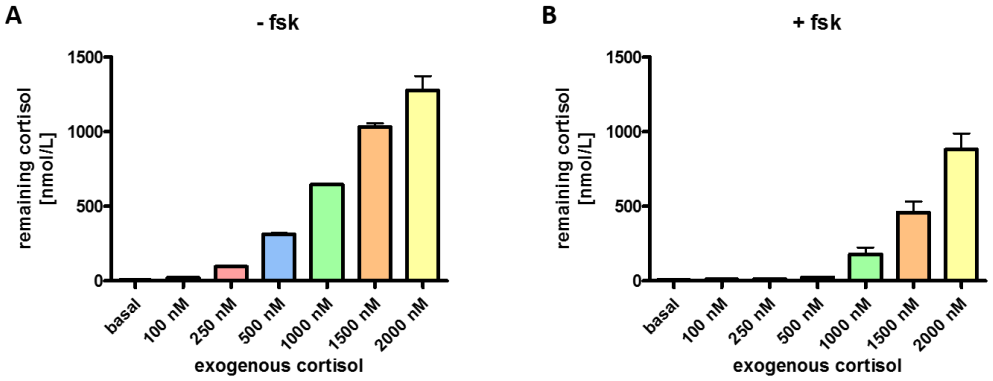

Figure 4.16: **Activity of 11β-HSD2 in BeWo cells.** BeWo cells were treated with varying concentrations of cortisol **(B)** with and **(A)** without 100 μM forskolin for 24 h before the remaining cortisol was measured in the supernatant. Cortisol concentration is normalized to protein concentration of BeWo cells, n=4.

4.2.4 Expression of Transporter Proteins P-gp and BCRP in BeWo Cells

I also attempted to determine the effect of forskolin on P-glycoprotein (ABCB1) and BCRP (ABCG2) expression. P-pg and BCRP mRNAs were expressed in the BeWo cells and were both significantly upregulated by forskolin treatment compared to control-treated cells (Figure 4.17). However, further analysis of P-gp in subsequent chapters was not considered suitable due to its low level of expression (mean Ct value was 34.4).

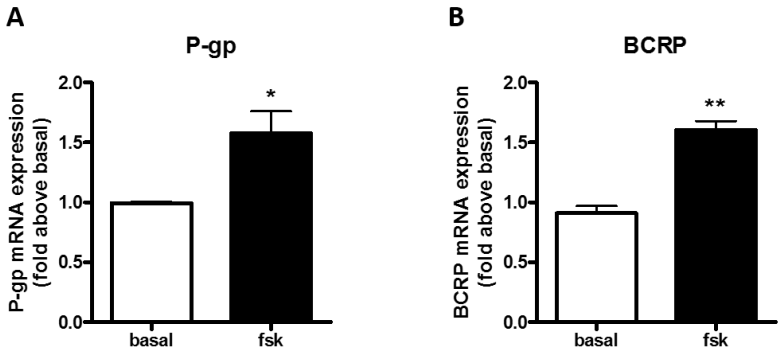

Figure 4.17: **mRNA expression of P-gp and BCRP in BeWo cells after forskolin treatment.** BeWo cells were treated with 100 μM forskolin for 24 h before mRNA was isolated and a qRT-PCR for P-gp **(A)** and BCRP **(B)** was performed. qRT-PCR was normalized to 18S rRNA expression, n=3, data are expressed as mean values ± SEM, t-test: $p<0.05$ (=*), $p<0.01$ (=**).

4.3 Discussion of Chapter 4

The data of this chapter show that BeWo cell fusion and differentiation can be successfully established and monitored upon forskolin stimulation (Figure 4.18 for summary) in agreement with previous studies [Ringler and Strauss, 1990; Wice et al., 1990; Sullivan, 2004; Orendi et al., 2011]. BeWo cells morphologically fuse after forskolin treatment which is associated with an upregulation of the fusogenes Syncytin-1 and Syncytin-2. In parallel, biochemical differentiation of the BeWo cells is activated. During this process, endocrine activity of BeWo cells is established as shown by the upregulation of the hormones hCG and progesterone. Furthermore, the expression of the hormone CRH and its receptors CRH-R1 and CRH-R2 is increased after forskolin treatment which might increase cell responsiveness to autocrine actions of CRH. Also, the glucocorticoid barrier enhances upon trophoblast differentiation as the enzyme 11β-HSD2 and cortisol transporter molecules are upregulated. Regarding glucocorticoid action, the two cortisol-binding receptors gluco- and mineralocorticoid receptor are expressed in the BeWo cells, the latter being downregulated during differentiation. This might act as a counterregulatory mechanism to protect the cells against inappropriate MR-stimulation.

These data demonstrate that BeWo cells are an appropriate model to investigate the trophoblast biological functions of 11β-HSD2, its cross-talk with CRH and glucocorticoids and placental cortisol metabolism (see chapters 5 and 6).

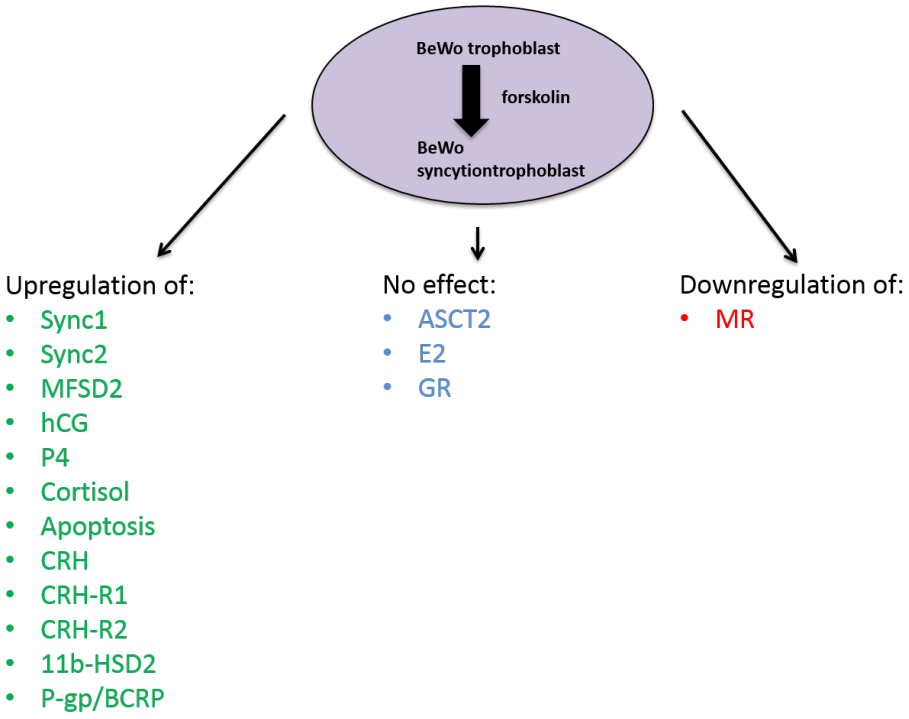

Figure 4.18: **Summary of forskolin effects on BeWo cell biology.**

A very important process in the lifespan of a placenta is the differentiation of cytotrophoblasts into a syncytium. *In vivo*, the cytotrophoblast cells start their cellular differentiation and ultimately fuse with the adjacent syncytiotrophoblast. During this process, the biochemical characteristics of the cells change, e.g. the syncytiotrophoblast produces large amounts of hormones such as hCG, progesterone and placental lactogen [Reis et al., 2001]. Hence, a key task of this experimental approach was to show that the BeWo cells are able to fuse as well as undergo biochemical differentiation.

In order to monitor the fusion process of BeWo cells, three different methods (brightfield microscopy with nuclei staining, fluorescent membrane staining, fluorescent cell tracker staining) were used since the detection of the fusion process is challenging. Brightfield images with a DAPI-nuclei staining is the easiest method and it lets presume that in my experiment BeWo cells have fused after forskolin treatment in some areas shown by a more dense accumulation of nuclei. However, staining of the cell membrane is a more appropriate method since it is able to demonstrate the loss of the cell membranes which is a characteristic of cell fusion. Coutifaris et al. [1991] investigated the E-Cadherin expression in primary isolated cytotrophoblasts as well as in BeWo cells and in non-fusing JEG-3 cells. They showed that in BeWo cells, treated with 8-bromo cyclic AMP, and in primary cells the E-Cadherin disappeares in fused cells, whereas in JEG-3 cells no difference in E-Cadherin staining is detectable. In agreement with this, 48 h of forskolin treatment in my experiment also led to disappearance of E-Cadherin staining in areas of fused cells. Another method to detect cell fusion was based on the labeling of two BeWo cell populations with different cell tracker dyes and their subsequent proliferation and differentiation after mixing the two populations. During the fusion process, cytoplasmic content is exchanged between the fused cells and in case of using the cell tracker dyes Dil (red fluorescence) and DiO (green fluorescence), fused cells are detectable as double-fluorescent yellow (overlay of red and green) stained cells as it was in my experiment after treating the BeWo cells with forskolin. Borges et al. [2003] and Wang et al. [2014a] used a comparable cell tracker dye method to show the fusion in BeWo, JEG-3, JAR cells and in primary trophoblast cells. Similarly to my results, they observed double-stained areas in BeWo cell and primary trophoblast cell populations, but not in JEG-3 nor JAR cells.

Borges et al. [2003] showed only minimal BeWo cell fusion after 24 h of 50 μM forskolin treatment which is in agreement with my observation. After 48 h of forskolin treatment, they detect considerably more fused cells than after 24 h. Also, Kudo et al. [2003a] showed maximal fusion of BeWo cells after 100 μM forskolin

treatment for 48 h. Taken these results together, it can be concluded that the fusion process of BeWo slowly progresses for the first 24 h of forskolin treatment, but 48 h of forskolin treatment is required to allow significant fusion of BeWo cells to be detected consistently.

Important molecules involved in the fusion process of trophobast cells are the fusogenes Syncytin-1 and Syncytin-2 with their receptors. Syncytins and their receptors play a fundamental role in the fusion process of cytotrophoblasts into the syncytium in the placenta [Pötgens et al., 2004] as well as in fusion of primary cells and cell lines (see subchapter "'Synctin-1 and Syncytin-2"' in chapter 1.2.5.3).

Several groups have shown that BeWo cells express Syncytin-1 and -2 and that forskolin treatment upregulates their expression [Kudo and Boyd, 2002; Vargas et al., 2009]. Similarly, my data confirmed an upregulation of Syncytin-1 and -2 on mRNA level after forskolin treatment. Interestingly, it showed a higher upregulation of Syncytin-1 and -2 occurs after 24 h than after 48 h of forskolin treatment. Thus it seems that, after 24 h of forskolin treatment the fusogenes Syncytin-1 and -2 are highly upregulated, but the physical fusion process is at its early stages and requires at least 48 h for detectable fusion.

Regarding the fusogene receptors ASCT2 (Syncytin-1 receptor) and MFSD2 (Syncytin-2 receptor), mRNA expression of ASCT2 showed a trend to be downregulated after 24 h of forskolin, whereas MFSD2 mRNA was upregulated. Similar to these findings, Kudo et al. [2004] and Kudo et al. [2003c] detected a downregulation of ASCT2 mRNA after forskolin treatment in BeWo cells. Furthermore, Toufaily et al. [2013] observed an upregulation of MFSD2 mRNA and protein in forskolin-treated BeWo cells. My data concerning the syncytins and their receptors appear to be in agreement with the previously published literature.

Viability and apoptosis studies on BeWo cells have shown that forskolin treatment affects cell viability and apoptosis [Al-Nasiry et al., 2006; Chen et al., 2011]. Compared to the *in vivo* situation in the placenta, this treatment with a possibly toxic chemical substance is a disadvantage of the BeWo cell system. On the other hand, the most representative system available are primary trophoblast cells or placental explants, but as mentioned in the previous chapter, placental explants are characterized by poor reproducibility. Similar issues might be present when performing experiments with primary trophoblast cells, however this might be less present as primary cells consist of one homogenous cell type.

As differentiation processes seem to be linked to apoptotic processes (Chapter

1.2.6.3), apoptosis in BeWo cells after forskolin treatment was investigated. Areas of fused cells displayed activated caspase-3/7 which indicates that the differentiation process is linked to apoptotic processes in BeWos cells similar to the situation *in vivo*. In my staining experiments, longer incubation with forskolin (when comparing 24 h with 48 h of forskolin treatment) activated more caspase-3/7. Chen et al. [2011] also showed an activation of caspase-3 as early as after 24 h of treatment with 8-Br-cAMP. Because after 48 h of forskolin treatment detached floating BeWo cells were observed in addition to apoptotic processes, forskolin treatment was set to 24 h for all subsequent experiments. This experimental design ensured that the BeWo cells were viable during other treatments and that results would be compromised as little as possible by apoptosis. Moreover, the observed downregulation of forskolin-induced expression of Syncytin-1 and -2 as well as 11β-HSD2 after 48 h compared to 24 h of forskolin stimulation might result from the apoptotic processes.

hCG is the hormone which is most often used as a marker for biochemical differentiation of trophoblast cells in many studies which used primary trophoblast cells or trophoblast cell line systems. In my experiments, incubation of BeWo cells with forskolin increased the hCG secretion by 8-fold. Likewise, Chou et al. [1978], Futamura et al. [1987] and Delidaki et al. [2011] showed that stimulation of cAMP increases the hCG production in BeWos. Ringler et al. [1989b] treated primary trophoblast cells with forskolin which induced hCG production. Even though, primary trophoblast cells are capable of secreting hCG without stimulation of cAMP with forskolin or other cAMP-stimulating agents, this result reveals that forskolin has the same effect in primary cells as in trophoblast cell lines. Thus, BeWo cells seem to behave similar to primary cells after being treated with forskolin.

Interestingly, it has been shown by Al-Nasiry et al. [2006] and Orendi et al. [2010] that the fusion process of cells is uncoupled from the biochemical differentiation. Al-Nasiry et al. [2006] showed that in the non-fusing trophoblast cell line JEG-3, forskolin stimulation leads to an increase in hCG production while no fusion is occuring. Orendi et al. [2010] observed an hCG production in forskolin-stimulated BeWo cells after blocking the cell fusion. Similarly to these findings, my data showed that fusion events are barely detectable after 24 h of forskolin treatment whereas hCG production was already increased by 8-fold suggesting that biochemical differentiation is progressing more rapidely in BeWo cells. Another possibility is that hCG production might accelerate the BeWo cell fusion process in an autocrine manner. In support of this, Dhar et al. [2004] showed that after blocking the endogenous hCG with an antibody, primary cytotrophoblast fusion is impaired. The expression of the

hCG receptor in placental tissue was shown by Reshef et al. [1990], and Shi et al. [1993] also showed that hCG regulates cytotrophoblast differentiation in primary cells.

The hormones progesterone (P4) and estradiol (E2) have multiple very important functions in placentation and fetal development and growth (see chapter 1.2.7.2) and therefore, they would be important marker molecules for endocrine functionality. Progesterone secretion increased after forskolin treatment in BeWo cells. This finding is consistent with the result from Nulsen et al. [1989] and Maldonado-Mercado et al. [2008]. They showed that stimulation of cAMP upregulates progesterone secretion in BeWo cells. Progesterone might act in an autocrine manner on BeWo cells as these are shown to express different progesterone receptors [Zachariades et al., 2011]. Also, a cross-talk of hCG with P4 might occur in the BeWo cell line since Dhar et al. [2004] showed that blocking of hCG by using an hCG antibody decreased the production of progesterone in primary trophoblast cells. So it might be possible that hCG is an autocrine inducer of P4 production which could explain the similar secretion pattern of both hormones after forskolin treatment. Furthermore, progesterone then might have a positive effect on cell fusion as Noorali et al. [2009] detected the upregulation of Syncytin-1 by progesterone in a trophoblast cell line.

Cortisol can not be produced by trophoblasts because the enzymes 21-hydroxylase (CYP21A2) and 11β-hydroxylase (CYP11B1) are absent [Sullivan, 2004]. Interestingly, there seems to be a different setting in BeWo cells. My result showed a small increase of cortisol production after forskolin treatment. Jeschke et al. [2007] also observed that BeWo cells can produce cortisol. They did not treat the cells with forskolin, but they observed a small increase in cortisol production after treatment with glycodelin A N-glycan. Nevertheless, it shows that BeWo cells seem to be capable of producing small amounts of cortisol.

Investigations of additional molecules showed that BeWo cells expressed CRH and both receptor (CRH-R1 and -R2) mRNA and all of which were upregulated after forskolin treatment. Similar to my studies, Chen et al. [2013] showed that 8-Br-cAMP treatment leads to an upregulation of CRH and both CRH-R1 and -R2 mRNAs in BeWo cells. Furthermore, this is identical to the up-regulation of CRH by forskolin and 8-Br-cAMP in primary trophoblast cells [Cheng et al., 2000]. Like the hormones hCG and progesterone, CRH might have an effect on the fusion of the BeWo cells. Chen et al. [2013] showed that treatment of BeWo cells with CRH leads to an upregulation of Syncytin-1 and increased the fusion process. On the

other hand, even though Fahlbusch et al. [2012] could demonstrate a CRH-induced upregulation of Syncytin-1 in primary trophoblast cells, they did not detect an associated increased syncytialisation process. These discrepancies indicate that the role of CRH is unclear.

Placental tissue is responsive to glucocorticoids [Giannopoulos et al., 1983; López Bernal et al., 1984] and both receptors, gluco- and mineralocorticoid receptor, are expressed [Hirasawa et al., 2000; Driver et al., 2003; Patel et al., 2003; Chan et al., 2003]. In my experiments, BeWo cells expressed the glucocorticoid and the mineralocortiocid receptor mRNA, and interestingly the latter was downregulated after forskolin treatment, whereas the glucocorticoid receptor was not affected. The downregulation of the mineralocorticoid receptor seems to be a mechanism which leads to protection of the cells against inappropriate stimulation of MR by cortisol in their differentiated state (i.e. syncytium-like state). Mark and Waddell [2006] detected a low expression of GR in BeWo cells which prompted them to use a GRα expression vector in their studies.

The syncytiotrophoblast of the placenta highly expresses 11β-HSD2 and this successfully inactivates cortisol, protecting the fetus from excess cortisol [Krozowski et al., 1995]. This is considered to be the main function of placental 11β-HSD2 and has been subject to extensive research over the past years.

After differentiation (treatment with forskolin) the BeWo cells expressed considerably higher levels of 11β-HSD2 mRNA and protein than the undifferentiated cells suggesting that BeWo cells decrease cortisol action in their differentiated state. This pattern seems to be similar to the placental *in vivo* situation where the syncytiotrophoblast layer expresses high amounts of 11β-HSD2 [Krozowski et al., 1995]. In addition, it has been shown before that stimulation of cAMP induces 11β-HSD2 expression in primary trophoblast cells [Sun et al., 1998]. Thus, the regulation of 11β-HSD2 in BeWo cells seems to be comparable to other cell systems. Náray-Fejes-Tóth and Fejes-Tóth [1996] investigated the localisation of 11β-HSD2 with a GFP-fusion protein and discovered its localisation in the cytosol, more specifically as a reticular network, but no expression of the enzyme in the membrane nor in the nucleus of CHO cells. In contrast, Shimojo et al. [1997] observed a proportion of 11β-HSD2 localized in the nucleus in kidney sections. So, the localisation of the enzyme seems to be celltype-specific and the BeWo cells displayed a cytoplasmic localisation of the 11β-HSD2.

11β-HSD2 also influences the local glucocorticoid action within the tro-

phoblast cells. By inactivating cortisol into cortisone, less cortisol molecules are available within the syncytiotrophoblast to bind to the gluco- as well as mineralocorticoid receptor and consequently, less glucocorticoid action takes place. Thus, 11β-HSD2 limits the local glucocorticoid action within the placenta. In mineralocorticoid-target tissue, the main function of 11β-HSD2 is considered to be the protection of the MR against cortisol binding [Edwards et al., 1988; Funder et al., 1988]. Potentially this function of 11β-HSD2 within the placenta might also be of high importance. Driver et al. [2003] characterized the placenta as a mineralocorticoid-target tissue due to its expression of a functional MR. Especially, the observation that forskolin downregulates the MR expression during BeWo cell differentiation (see above) combined with the high upregulation of 11β-HSD2 after forskolin treatment might provide clues that during trophoblast differentiation, the system prevents inappropriate effects of cortisol via MR.

Another important player in regulating glucocorticoid action are transporter molecules (see chapter 1.1.6). Both ABC transporter molecules P-glycoprotein (ABCB1) and BCRP (ABCG2) are expressed in the placenta [Ceckova et al., 2006]. It has been shown that the transporter P-glycoprotein can transport cortisol molecules out of the cell. The BeWo cells did express P-glycoprotein mRNA, but due to its low expression, investigation of this transporter was not pursued further. Even though Utoguchi et al. [2000] and Mark and Waddell [2006] observed a functional P-glycoprotein transporter in BeWo cells, several other groups did report a low expression of P-glycoprotein in BeWo cells [Atkinson et al., 2003; Evseenko et al., 2006]. In addition, Ceckova et al. [2006], Magnarin et al. [2008] and Crowe and Keelan [2012] did not detect any P-glycoprotein in BeWo cells.

The efflux transporter molecule BCRP is predicted to transport cortisol [Mares-Sámano et al., 2009] and its mRNA expression was very high in BeWo cells and was further upregulated by forskolin treatment. Consistent with this result, Ceckova et al. [2006] showed a high expression of BCRP in BeWo cells and Evseenko et al. [2006] showed that BCRP mRNA increases during primary trophoblast differentiation. In the subsequent chapters, only analysis of the transporter BCRP was further pursued.

Characterization of the BeWo cells revealed that once the cells differentiate, they elevate hormone secretion suggesting an increased endocrine activity which mimics the endocrine capacity of the placental syncytiotrophoblast. They also secrete more steroid hormones whereby a more progestogenic environment is estab-

lished. Moreover, they become more resilient in dealing with excess cortisol by highly upregulating 11β-HSD2 which resembles the *in vivo* situation where the syncytiotrophoblast expresses high amounts of this enzyme. Interestingly, they become less responsive to cortisol-induced MR effects by downregulating MR and this could be the subject of future experiments characterizing how the placenta protects itself against MR effects and how these dynamics are altered during placental diseases.

Chapter 5

Role of 11β-HSD2 in BeWo Cell Biology

In this chapter, the role of 11β-HSD2 in trophoblast biology using the choriocarcinoma cell line BeWo was investigated. The enzyme 11β-HSD2 is the main component of the so-called glucocorticoid barrier in the placenta preventing excess cortisol to reach the fetus [Benediktsson et al., 1997]. Maternal cortisol levels are five to ten times higher than fetal levels suggesting a very efficient catabolism of active cortisol to inactive cortisone by 11β-HSD2 [Beitins et al., 1973; Campbell and Murphy, 1977]. Through this mechanism, the fetus is protected against maternal cortisol, which in excess can lead to detrimental effects during fetal development and growth [Reynolds, 2013a]. However, it has been shown that the expression and activity of 11β-HSD2 is decreased in various placenta-related diseases such as pre-eclampsia, IUGR, and HELLP. Furthermore, molecules which are able to downregulate 11β-HSD2 have been identified and many of them are involved in the pathogenesis of these placenta-related diseases. Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 reduce 11β-HSD2 activity [Chisaka et al., 2005; Kossintseva et al., 2006], and hypoxia is involved in the downregulation of 11β-HSD2 [Homan et al., 2006]. Thus, many different pathological conditions lead to the downregulation of the important enzyme 11β-HSD2 and the effects of this pathological feature on the placental biology has not been investigated so far.

The aim of this chapter was to investigate which putative effects the downregulation of 11β-HSD2 has in trophoblast biology including cell viability and apoptosis, endocrine function, and expression of proteins involved in the stress response and glucocorticoid action molecular machinery. This possibility of altering trophoblast function was addressed in this chapter by choosing an siRNA approach to downreg-

ulate 11β-HSD2 in BeWo cells.

Furthermore, the potential regulation of 11β-HSD2 by CRH was investigated. In the placenta, CRH and cortisol are interconnected via a positive feedback loop which is in contrast to the negative feedback regulation of cortisol on hypothalamic CRH production [Robinson et al., 1988]. As 11β-HSD2 is an important modulator of active cortisol availability within cells, its expression and activity has the potential to influence the positive feedback loop between cortisol and CRH. So it might be possible that CRH can influence the expression and activity of 11β-HSD2 in order to fine-tune this dynamic equilibrium of cortisol and CRH concentration.

BeWo cells were chosen as a suitable model because, as shown in chapter 4.2.3, they express high endogenous levels of 11β-HSD2 (Figure 4.14 and 4.15). Moreover, its expression mimics the placental *in vivo* situation as differentiation of BeWo cells leads to a substantial upregulation of 11β-HSD2 which resembles the higher expression in the syncytiotrophoblast compared to cytotrophoblasts (Figure 3.13 C, [Krozowski et al., 1995; Driver et al., 2001]).

5.1 Knockdown of 11β-HSD2 in BeWo Cells

To investigate the role of 11β-HSD2 in BeWo cells, they were treated with an siRNA which downregulates the 11β-HSD2 expression. This siRNA, which targets 11β-HSD2 mRNA, is referred to as siHSD2 in all subsequent sections.

siRNA Transfection in BeWo Cells

Before investigating conditions that downregulate 11β-HSD2 in BeWo cells, the siRNA transfection capacity of BeWo cells was tested using a lipid-based transfection of fluorescently-labelled non-targeting siRNA. The widespread red staining (fluorescently-labelled siRNA) in Figure 5.1 shows a successful transfection of BeWo cells and so this experimental condition was used for the subsequent experiments.

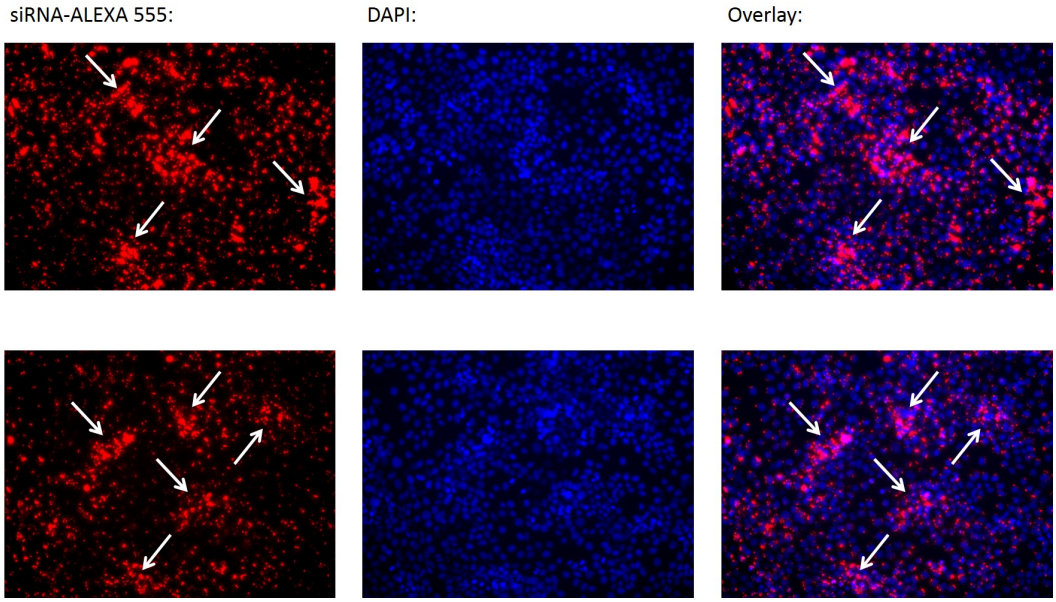

Figure 5.1: **BeWo cells transfected with ALEXA555-labelled non-targeting siRNA.** BeWo cells were transfected with 15 nM fluorescently-labelled (ALEXA-555) siRNA for 24 h (left images), which is shown in red and the nuclei were stained with Hoechst (middle images), which is shown in blue. Overlay (right images), representative images are shown.

5.1.1 Knockdown of 11β-HSD2 mRNA and Protein

Following establishment of conditions required to transfect BeWo cells with siRNA (see above), the kinetics of the activity of siHSD2 was evaluated. BeWo cells were treated with siHSD2 for 24, 48, and 72 h. Figure 5.2 shows that siHSD2 reached the maximum effect 24 h after transfection, i.e. within 24 h siHSD2 induced a significant inhibition of 11β-HSD2 mRNA by 77.7% (Figure 5.2 A). After 48 and 72 h of siHSD2 treatment, 11β-HSD2 mRNA expression was significantly reduced by 73.1% and by 50.2%, respectively (Figure 5.2 B and C).

In addition to downregulation of mRNA, I also measured putative effects on the protein level. Similarly to 11β-HSD2 mRNA, 11β-HSD2 protein was also downregulated on protein level for up to 72 h (Figure 5.2 D).

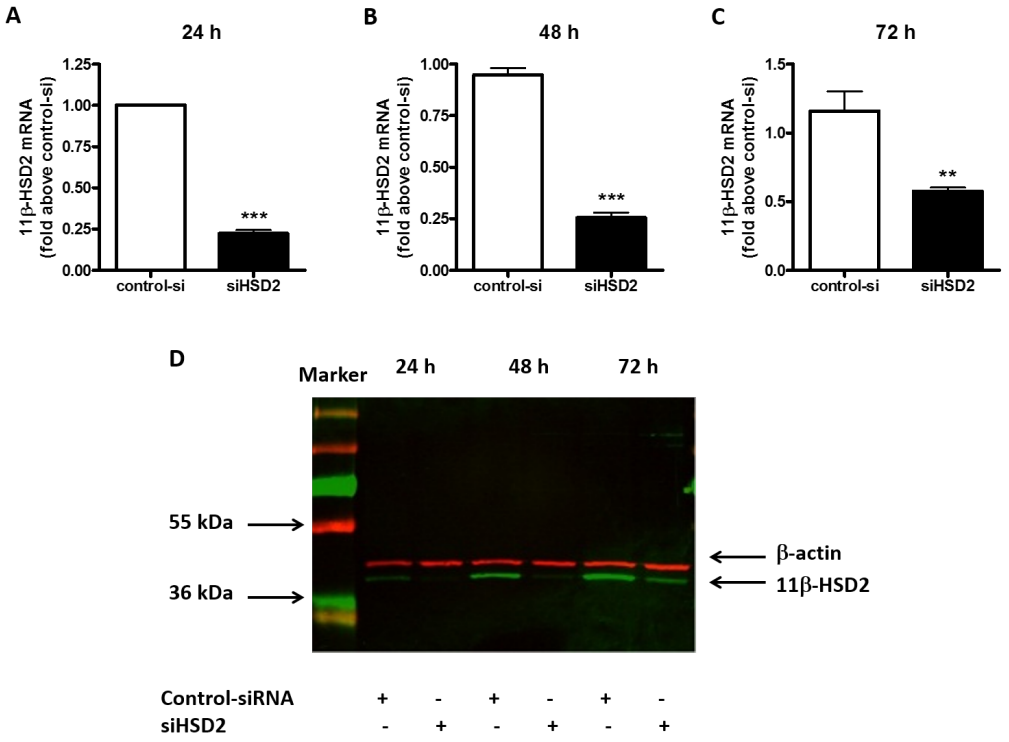

Figure 5.2: **11β-HSD2 knockdown in BeWo cells.** BeWo cells were transfected with 15 nM siHSD2 or 15 nM control-siRNA (= control-si) for 24 h, 48 h and 72 h before qRT-PCR and western blot for 11β-HSD2 were performed. **(A, B, C)** mRNA level of 11β-HSD2 after siRNA treatment, **(D)** protein level of 11β-HDS2 after siRNA treatment. **(A, B, C)** qRT-PCR data was normalized to 18S rRNA expression, data are expressed as mean values ± SEM, n=3, t-test: $p<0.01$ (=**), $p<0.001$ (=***). **(D)** β-actin (red band) was used as a loading control in the western blot, a representative blot is shown.

Having established conditions required to achieve effective inhibition on 11β-HSD2 mRNA and protein expression, I investigated the knockdown of 11β-HSD2 during differentiation of the BeWo cells. The following protocol of transfection with forskolin treatment was used in the subsequent experiments: (1) 24 h of siRNA treatment with subsequent 24 h forskolin treatment and (2) 48 h of siRNA treatment with subsequent 24 h forskolin treatment (for treatment protocol see Figure 2.3 (1b) in chapter Material and Methods) as both time points showed a successful downregulation of 11β-HSD2 on mRNA and protein level (Figure 5.2).

Figure 5.3 A shows the downregulation of 11β-HSD2 mRNA after 24 h of siHSD2 treatment in un- and differentiated BeWo cells ($\pm$ 100 μM forskolin for 24 h). Forskolin led to a strong upregulation of 11β-HSD2 mRNA compared to undifferentiated cells as previously also shown in chapter 4.2.3 (Figure 4.14). In undifferentiated cells (without forskolin treatment), siHSD2 led to a significant decrease of 11β-HSD2 mRNA expression by 60% in the cells compared to control-siRNA treated cells. In differentiated cells (with forskolin treatment), the significant decrease in 11β-HSD2 mRNA was comparable (57%) after siHSD2 treatment. Forty-eight hours of siHSD2 treatment combined with forskolin treatment led to a similarly successful downregulation of 11β-HSD2 mRNA (data not shown).

Figure 5.3 B and C show the downregulation of 11β-HSD2 protein after 24 h and 48 h of siHSD2 treatment with or without a subsequent treatment with 100 μM forskolin for 24 h. Similar to the mRNA data, the siHSD2 led to a successful downregulation of 11β-HSD2 in un- and differentiated cells on protein level.

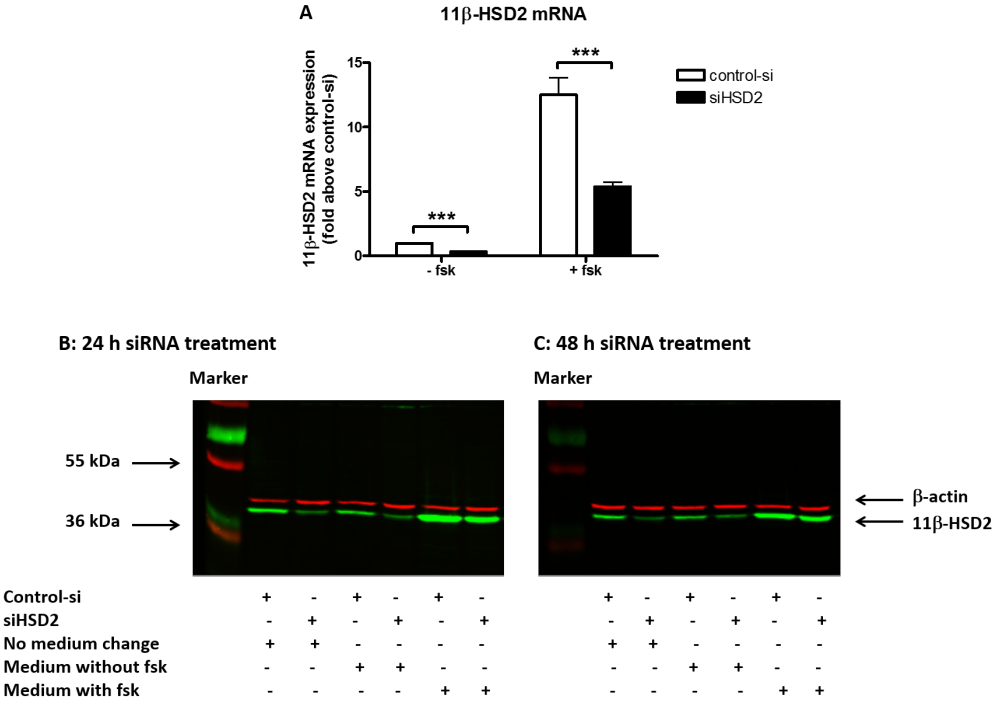

Figure 5.3: **11β-HSD2 knockdown in un- and differentiated BeWo cells.** BeWo cells were treated with siRNA (control-siRNA or siHSD2) for 24 h or 48 h with further incubation with and without 100 μM forskolin before qRT-PCR **(A)** and western blot **(B, C)** for 11β-HSD2 was performed. **(A)** qRT-PCR data was normalized to 18S rRNA expression, data are expressed as mean values $\pm$ SEM, 2-way ANOVA with Bonferoni post test: n=4, $p<0.001$ (=***). **(B, C)** β-actin (red band) was used as a loading control in the western blot, representative blots are shown.

5.1.2 11β-HSD2 Activity after Knockdown of 11β-HSD2

Following the successful effect of siHSD2 on 11β-HSD2 mRNA and protein expression, I determined whether this downregulation was associated with a decrease in 11β-HSD2 activity and the ability of cells to metabolize cortisol. As shown in chapter 4.2.3 (Cortisol Inactivation in BeWo cells), the activity of 11β-HSD2 was indirectly estimated by quantifying the remaining cortisol concentration in the supernatant after incubation of the cells with 500 nM cortisol for 24 h. The experimental protocol from the previous subchapter was repeated in the presence and absence of 500 nM cortisol (Figure 2.3 (1b) in chapter Material and Methods).

Results showed that undifferentiated BeWo cells metabolized 58% of the 500 nM of the exogenous cortisol, whereas the knockdown of 11β-HSD2 with siHSD2 significantly attenuated the metabolism of cortisol (Figure 5.4). The differentiated BeWo cells (with forskolin treatment) metabolized 96% of the exogenous cortisol reflecting the upregulation of 11β-HSD2 after forskolin treatment. When downregulating 11β-HSD2 with siHSD2, the differentiated BeWo cells metabolized significantly less cortisol and four times more cortisol remained in the cell culture supernatant. These results suggest that treatment of BeWo cells with siHSD2 reduced activity of 11β-HSD2 compared to control cells in both un- and differentiated BeWo cells.

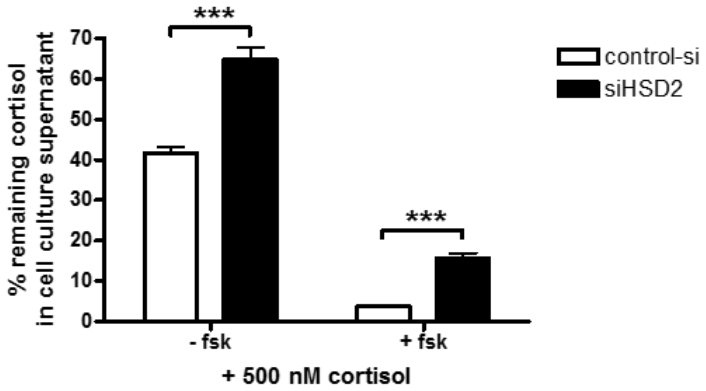

Figure 5.4: **Activity of 11β-HSD2 in BeWo cells after 11β-HSD2 knockdown.** BeWo cells were transfected with 15 nM control- or siHSD2-siRNA for 24 h with subsequent treatment with 500 nM cortisol with and without 100 μM forskolin for 24 h before measuring the remaining cortisol concentration in the supernatant. Cortisol concentration was normalized to protein concentration, data are expressed as mean values ± SEM, n=4, 2-way ANOVA with Bonferroni post test: $p<0.001$ (=***).

5.2 Role of 11β-HSD2 on Morphological Differentiation of BeWo Cells

The potential effect of 11β-HSD2 on morphological differentiation of BeWo cells was investigated. As differentiation of cytotrophoblast cells into syncytiotrophoblast is essential to maintain the physiological function of the syncytium, the mRNA expression of the fusogenic proteins Syncytin-1 and -2 and their receptors was examined.

Figure 5.5 shows that forskolin upregulated mRNA expression of Syncytin-1, Syncytin-2 and MFSD2, whereas mRNA expression of ASCT2 was slightly downregulated which is in agreement with the data obtained in chapter 4.1.2 (Figure 4.4 and 4.5). In undifferentiated cells (without forskolin treatment), knockdown of 11β-HSD2 did not change the mRNA expression of Syncytin-1, Syncytin-2 and their receptors MFSD2 and ASCT2 (Figure 5.5 A, B, C and D). In forskolin-induced differentiated cells, the mRNA expression of the fusogenic genes Syncytin-1 and -2 did not change after 11β-HSD2 knockdown (Figure 5.5 A and B), but the mRNA expression of their receptors ASCT2 and MFSD2 were significantly reduced by 21% and 43%, respectively, after downregulation of 11β-HSD2 (Figure 5.5 C and D).

These results suggested a pro-differentiating effect of 11β-HSD2 as a limited number of Syncytin receptors (after 11β-HSD2 downregulation) might impair the fusion process.

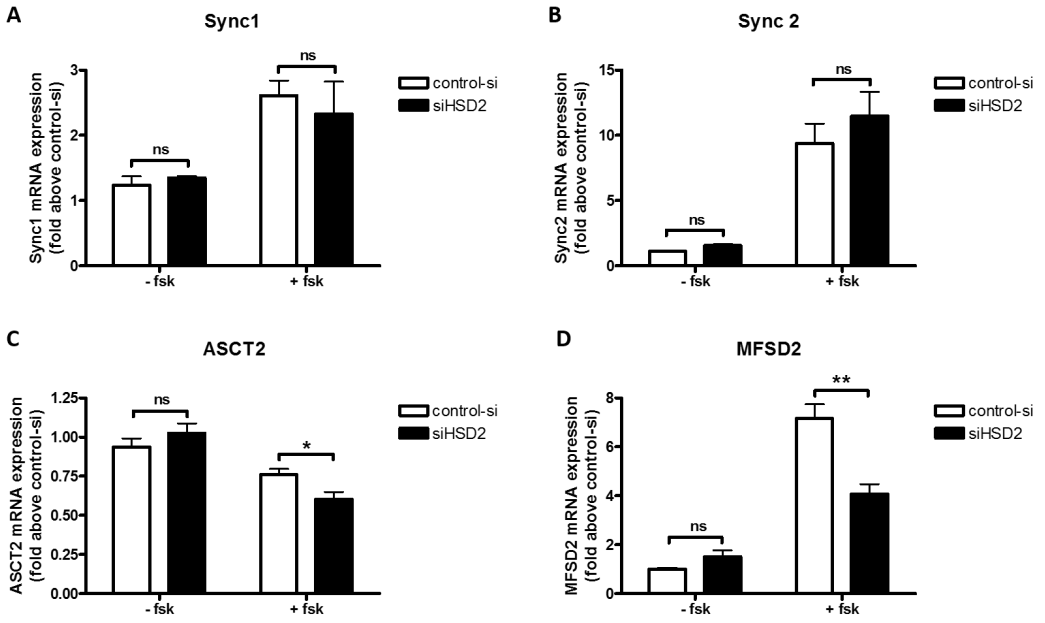

Figure 5.5: **Expression of Syncytin-1 and -2 and their receptors after 11β-HSD2 knockdown.** BeWo cells were pre-treated with siRNA for 24 h followed by a subsequent treatment for 24 h with and without 100 μM forskolin before mRNA extraction and determination of Syncytin-1 **(A)**, Syncytin-2 **(B)**, ASCT2 **(C)**, and MFSD2 **(D)** by qRT-PCR was performed. Data were normalized to 18S rRNA (in Syncytin-1 and -2) or to RPLP0 mRNA levels (in ASCT2 and MFSD2). Data are expressed as mean values ± SEM, n=4, 2-way ANOVA with Bonferroni post test: ns = non-significant, $p<0.05$ (=*), $p<0.01$ (=**).

5.3 Role of 11β-HSD2 on Hormone Production of BeWo Cells

BeWo differentiation is associated with increased production of hCG. The role of 11β-HSD2 in this process was further investigated.

BeWo cells were treated with siRNA (control-siRNA or siHSD2) for 24, 48, or 72 h and the secreted hCG was measured in the cell culture supernatant. Figure 5.6 shows that 11β-HSD2 knockdown led to a significant reduction in hCG production at all time points tested compared to control-treated cells (41%, 36%, and 31% decrease after 24, 48, and 72 h, respectively).

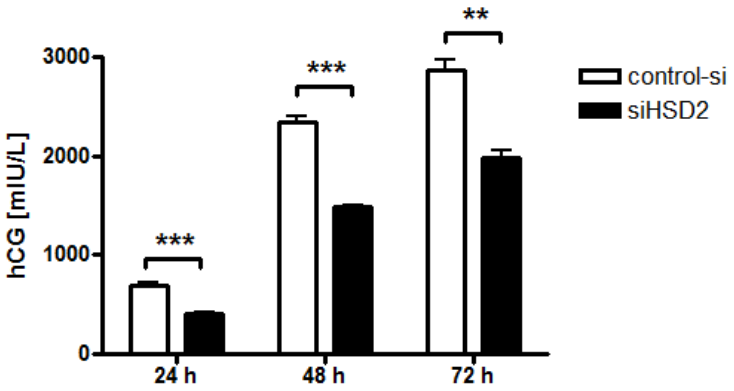

Figure 5.6: **hCG production of BeWo cells after 11β-HSD2 knockdown.** BeWo cells were cultured with siRNA for 24 h, 48 h, and 72 h before the concentration of secreted hCG was measured in the cell culture supernatant. hCG concentration was normalized to protein concentration of BeWo cells, data are expressed as mean values ± SEM, n=3, t-test: $p<0.01$ (=**), $p<0.001$ (=***).

To investigate the effect of 11β-HSD2 on hCG secretion in un- as well as differentiated cells, BeWo cells were treated with siRNA for 24 h with a subsequent incubation with fresh medium with or without 100 μM forskolin. The concentration of hCG was measured in the cell culture supernatant.

Forskolin treatment led to an increase in hCG secretion as previously shown in chapter 4.1.4 (Figure 4.8). In undifferentiated cells after 24 h of siHSD2 treatment, hCG concentration showed a trend to be decreased compared to control-siRNA treated cells, but just failed to reach statistical significance. In differentiated cells (with forskolin treatment), knockdown of 11β-HSD2 led to a significant de-

crease in hCG secretion by 48% compared to control-siRNA treated cells.

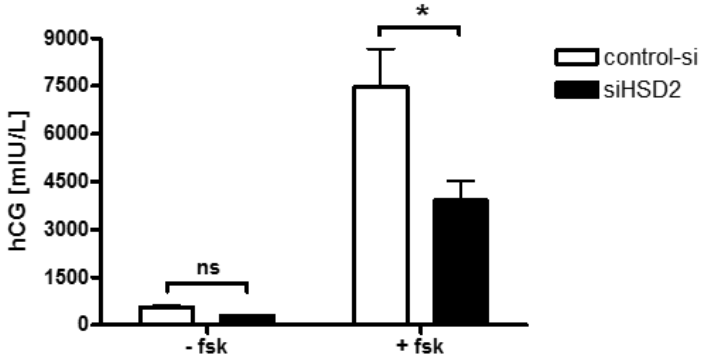

Figure 5.7: **hCG production of BeWo cells after 11β-HSD2 knockdown and forskolin treatment.** BeWo cells were cultured with siRNA for 24 h and subsequently incubated with or without 100 μM forskolin for 24 h before the concentration of secreted hCG was measured in the supernatant. hCG concentration was normalized to protein concentration of BeWo cells, data are expressed as mean values ± SEM, n=3, 2-way ANOVA with Bonferroni post test: ns = non-significant, $p<0.05$ (=*).

As previous results (Chapter 5.1.1) showed that knockdown of 11β-HSD2 was still evident after siHSD2 incubation for 48 h, in the subsequent experiments the incubation time with siRNA was increased to 48 h to test whether the prolonged downregulation of 11β-HSD2 might have a more profound effect on hCG production. Furthermore, forskolin treatment of 24 h was combined with 500 nM cortisol treatment to test the hypothesis whether an altered cortisol availability might be involved in the reduced hCG secretion associated with downregulation of 11β-HSD2.

A 48 h incubation of cells with siRNA had a similar inhibitory effect on hCG production (Figure 5.8 A) as the previous experiment with 24 h of siRNA incubation (Figure 5.7). In undifferentiated as well as in forskolin-treated differentiated cells, knockdown of 11β-HSD2 led to a significantly decreased hCG production by 52% and 53% compared to control-siRNA treated cells. Forskolin treatment led to an increase in hCG secretion as previously shown in chapter 4.1.4 (Figure 4.8). In contrast, cortisol treatment did not have an effect on hCG production suggesting that in this cellular system differentiation and hCG production occurs independently of any action of cortisol and 11β-HSD2 might target different signalling pathways.

In addition to measuring the hCG secretion, concentrations of progesterone (P4) and estradiol (E2) were also quantified in the cell culture supernatant to determine whether the observed hCG decrease after 11β-HSD2 knockdown was a specific effect. Forskolin treatment led to an increase of P4 secretion as previously shown in chapter 4.1.4 (Figure 4.8). In addition to hCG, progesterone also seems to be sensitive to 11β-HSD2 expression. In siHSD2-treated undifferentiated BeWos, P4 concentration showed a trend to be decreased compared to control-siRNA treated cells, although it failed to reach statistical significance. In differentiated BeWos, knockdown of 11β-HSD2 significantly decreased in P4 secretion by 47% compared to control-siRNA treated cells (Figure 5.8 B). Again, cortisol treatment did not change the siHSD2-induced decrease in P4 production.

Unlike hCG and progesterone, estradiol (E2) secretion was not affected by knockdown of 11β-HSD2 (Figure 5.8 C) suggesting that the effects of 11β-HSD2 downregulation on pathways regulating hCG and P4 production were selective. Again, cortisol treatment did not alter E2 production.

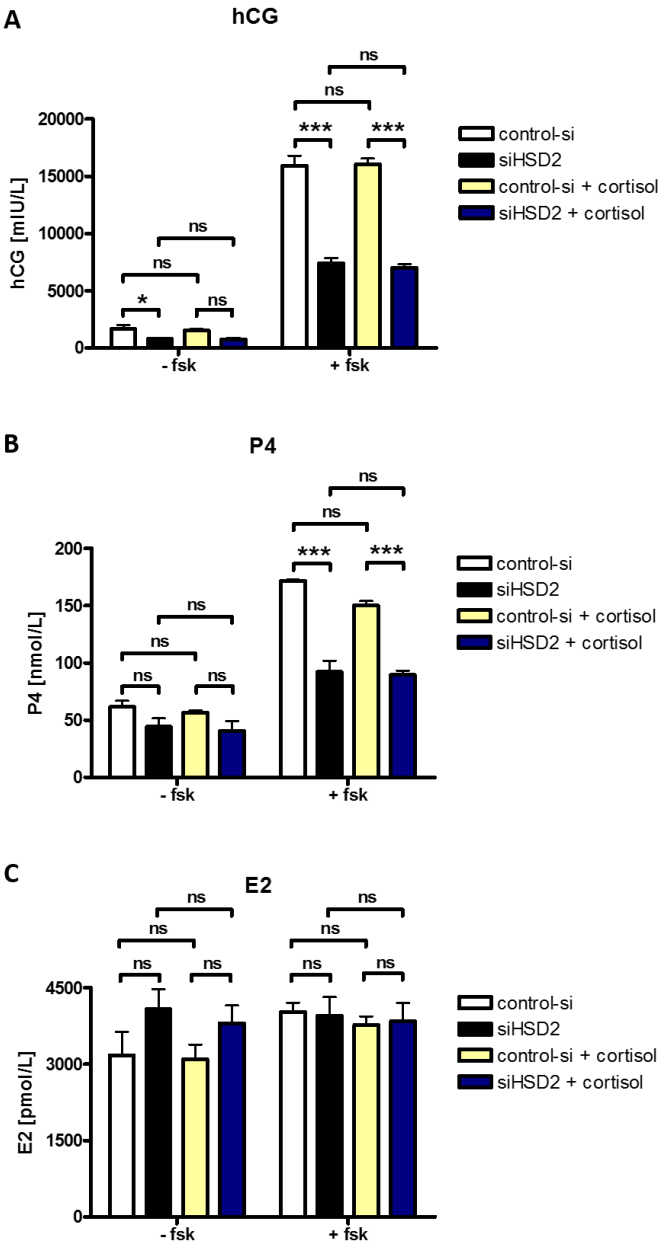

Figure 5.8: **hCG, P4, and E2 production of BeWo cells after 11β-HSD2 knockdown.** BeWo cells were cultured with siRNA for 48 h and subsequently incubated with fresh medium with and without 100 μM forskolin and/or 500 nM cortisol for 24 h before the concentration of secreted hCG **(A)**, P4 **(B)**, and E2 **(C)** was measured in the cell culture supernatant. hCG, P4 and E2 concentration were normalized to protein concentration of BeWo cells, data are expressed as mean values $\pm$ SEM, n = 3, 2-way ANOVA with Bonferroni post test: ns = non-significant, $p<0.05$ (=*), $p<0.001$ (=***).

5.4 Role of 11β-HSD2 on BeWo Cell Turnover

As the enzymes 11β-HSD1 and 11β-HSD2 have been linked to the regulation of cell proliferation and apoptosis in different cell types [Hundertmark et al., 1997; Lipka et al., 2004; Terao et al., 2013], such potential effects were investigated in the BeWo cells after knockdown of 11β-HSD2.

5.4.1 Role of 11β-HSD2 on BeWo Cell Viability

To investigate the role of 11β-HSD2 on BeWo cell viability, the MTS assay which measures formazan production by living cells was chosen. The measured signal (absorbance at 490 nm) is proportional to the living cell number. The BeWo cells were treated for 24 h and 48 h: the 24 h timepoint represents a treatment with control-siRNA or siHSD2 only for 24 h, whereas the 48 h timepoint represents a 24 h siRNA treatment followed by another 24 h with fresh medium with or without forskolin treatment (Figure 5.9 A).

Figure 5.9 B shows that siHSD2-treated cells were significantly less in number after 24 h of siRNA treatment compared to control-siRNA treated cells. A further 24 h incubation period with fresh medium with and without 100 μM forskolin treatment showed a trend (but not significant) to less cells in the siHSD2-treated compared to control-siRNA treated cells (Figure 5.9 C). This result suggests that initially downregulation of 11β-HSD2 leads to a reduced cell viability.

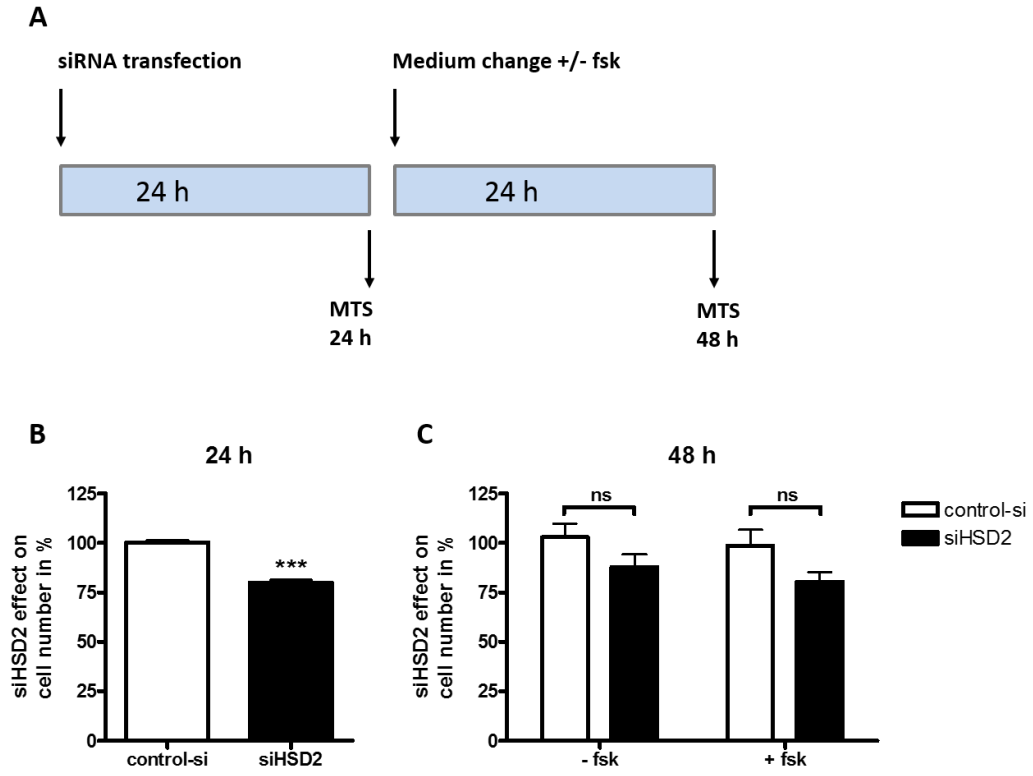

Figure 5.9: **Viability in BeWo Cells after 11β-HSD2 knockdown. (A)** Treatment protocol. BeWo cells were transfected with 15 nM control- or siHSD2-siRNA for 24 h **(B)** with and without a further treatment with 100 μM forskolin for 24 h **(C)** before measuring cell viability, n=3, data are expressed as mean values ± SEM, **(B)** t-test: p<0.001 (=***), **(C)** 2-way ANOVA with Bonferroni post-test: ns = non-significant.

5.4.2 Role of 11β-HSD2 on Apoptosis of BeWo Cells

These effects on cell viability led us to investigate the role of 11β-HSD2 on apoptosis. In undifferentiated BeWo cells, 11β-HSD2 was downregulated with siRNA for 24 h before employing detection of activated caspase-3/7 as a marker molecule for apoptosis. In parallel, to investigate the effect of 11β-HSD2 on apoptosis in differentiated BeWo cells, an incubation for another 24 h after knockdown with fresh medium with or without 100 μM forskolin was performed before measurement of activated caspase-3/7.

Qualitative Staining for activated Caspase-3/7

An increased signal for activated caspase-3/7 suggested that knockdown of 11β-HSD2 enhanced apoptosis after 24 h compared to control-siRNA treated cells (Figure 5.10). Therefore, the cell viability result (Figure 5.9 B), which showed a decrease in cell number after downregulation of 11β-HSD2, might be explained by an increased apoptosis after 11β-HSD2 downregulation.

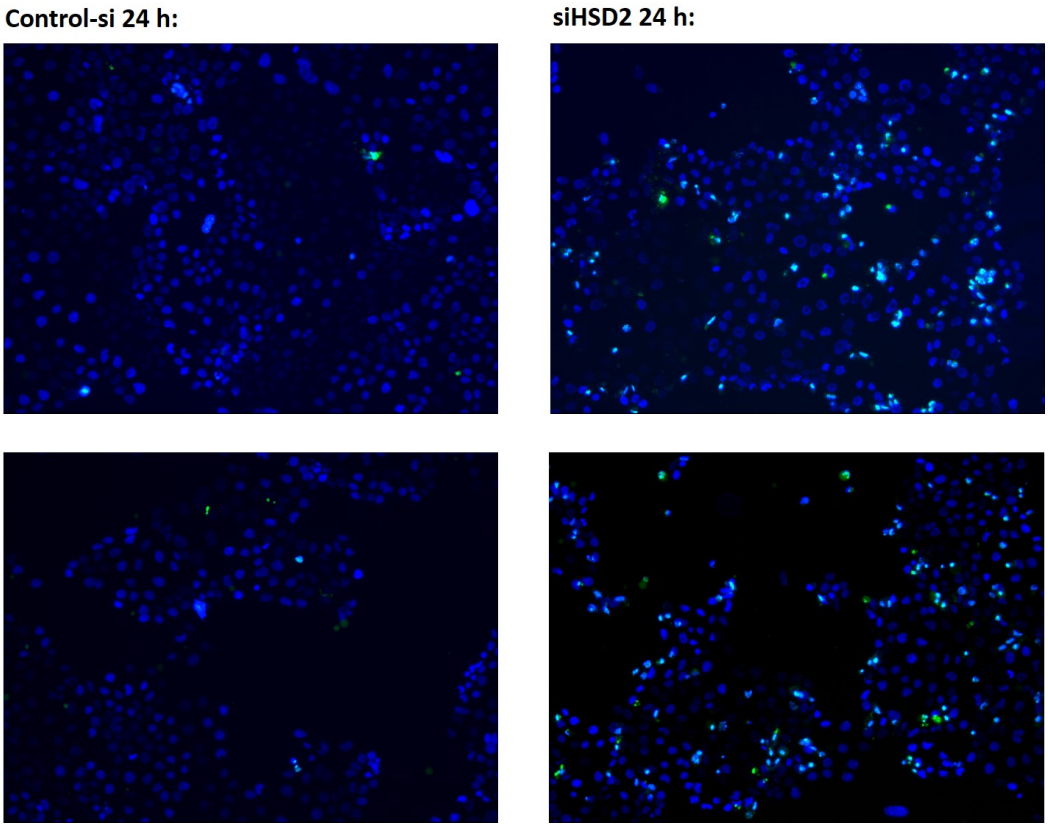

Figure 5.10: **Apoptosis of BeWo Cells after knockdown of 11β-HSD2 demonstrated by activated caspase-3/7 staining.** BeWo cells were treated for 24 h with either control-siRNA or siHSD2 before they were stained for activated caspase-3/7. Green staining = activated caspase-3/7 staining, nuclei are stained blue with DAPI, n=2, representative images are shown, each experimental condition is shown with two images.

Next, the 11β-HSD2 knockdown was followed by a further incubation with and without forskolin treatment (Figure 5.11). No difference in caspase-3/7 activation was detected between control-siRNA and siHSD2-treated cells in un- and differentiated cells suggesting that the initial activation of caspase-3/7 after 11β-HSD2 knockdown was attenuated by continuing cultivation of cells. The forskolin treatment led to more activated caspase-3/7 which is in agreement with the result from chapter 4.1.3 (Figure 4.6). In the immunofluorescent images, areas with a high cell density, detected by nuclei staining, displayed most activation of caspase-3/7.

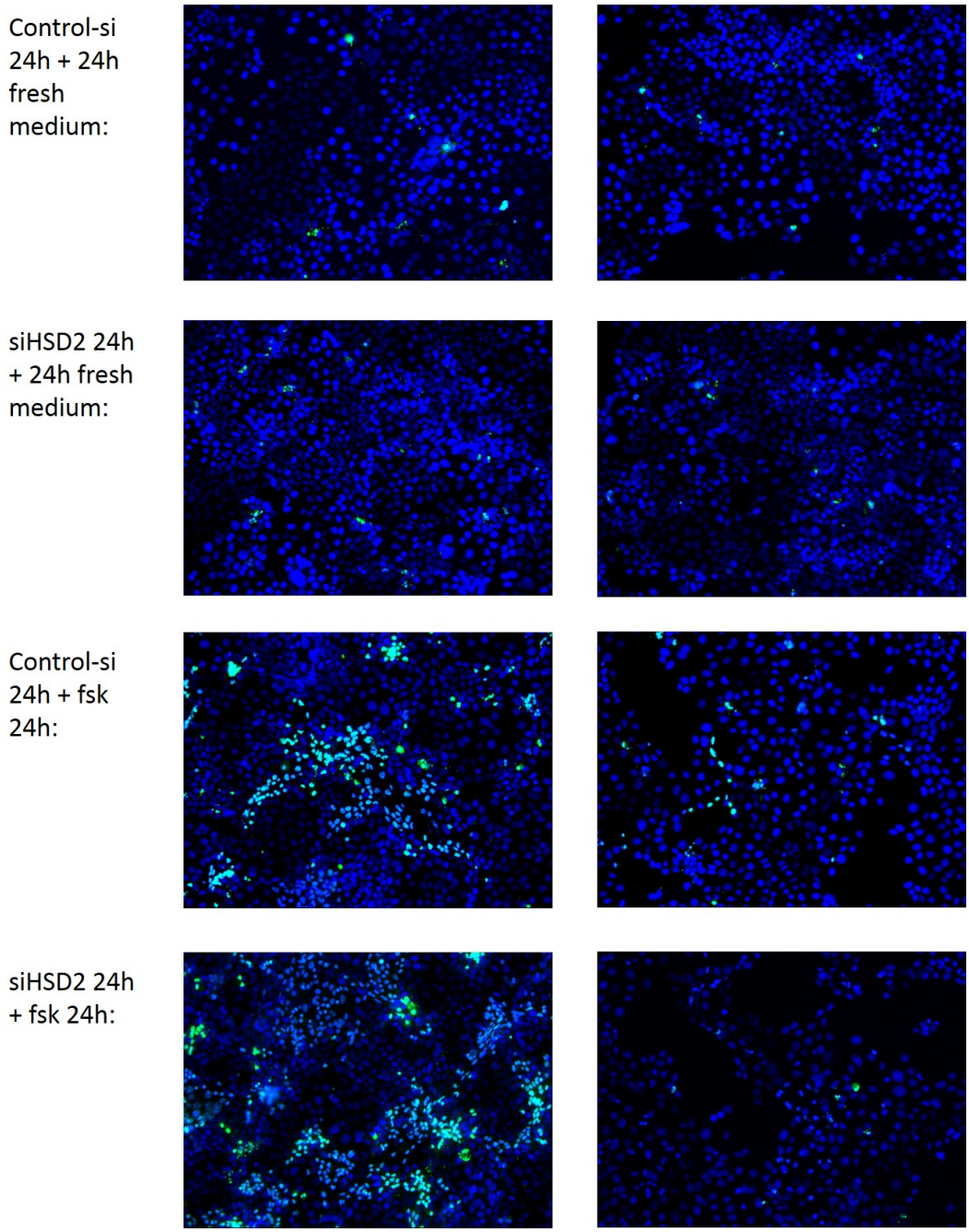

Figure 5.11: **Apoptosis of BeWo Cells after knockdown of 11β-HSD2 with forskolin treatment for further 24 h demonstrated by activated caspase-3/7 staining.** BeWo cells were treated for 24 h with either control-siRNA or siHSD2 and then for an additional 24 h with or without forskolin before they were stained for activated caspase-3/7. Green staining = activated caspase-3/7 staining, nuclei are stained blue with DAPI, n=2, representative images are shown, each experimental condition is shown with two images.

Quantitative Measurement of activated Caspase-3/7

The same protocol was applied in a different experimental approach aiming at quantifying the caspase-3/7 activation by measuring levels of a fluorescent substrate which is generated after being cleaved by activated caspase-3/7 (Figure 5.12 A).

Figure 5.12 B shows that BeWo cells treated with siHSD2 activated more caspase-3/7 than control-siRNA treated cells during the period of 24 h which is in agreement with the images obtained from the qualitative measurement of activated caspase-3/7 (Figure 5.10). This result suggests that 11β-HSD2 limits activation of caspase-3/7.

A further incubation of cells for 24 h led to a similar activation of caspase-3/7 in siHSD2-treated cells compared to control-siRNA treated cells in un- and differentiated BeWo cells (Figure 5.12 C). Forskolin treatment led to increased activation of caspase-3/7 which is in agreement with the result above and the result from chapter 4 (Figure 4.7) which showed an activation of caspase-3/7 after forskolin treatment.

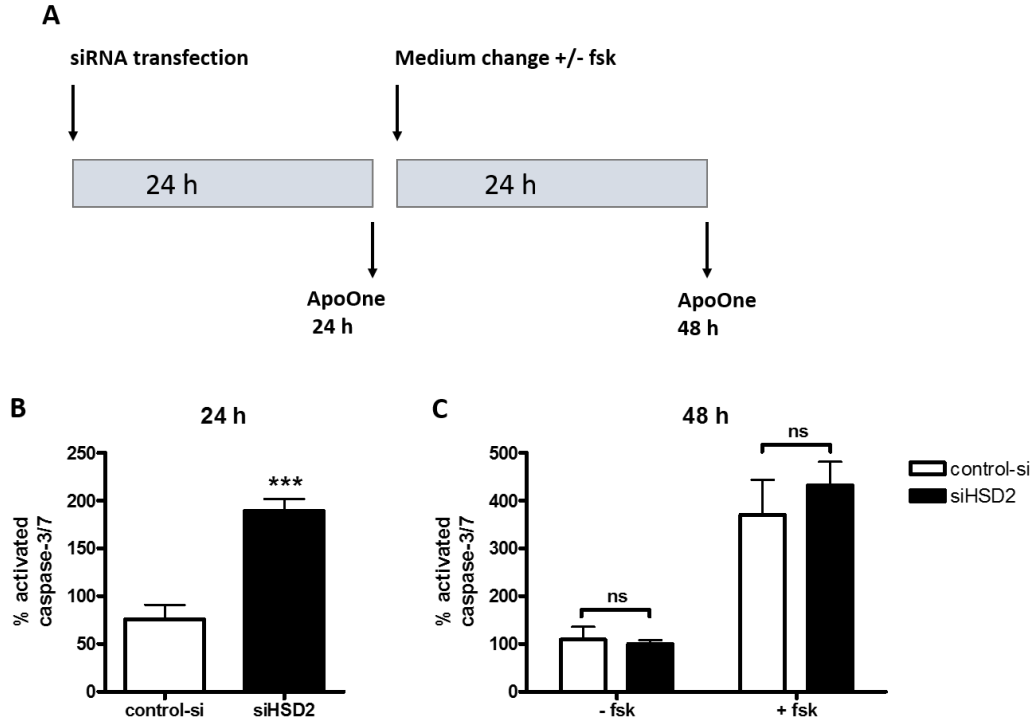

Figure 5.12: **Caspase-3/7 activation in BeWo cells after 11β-HSD2 knock-down.** **(A)** Treatment protocol. BeWo cells were treated with 15 nM siRNA for **(B)** 24 h or **(C)** for a further 24 h with or without 100 μM forskolin treatment before detecting activated caspase-3/7 with the ApoOne Assay which measured a fluorescent cleaved substrate. n=3, data are expressed as mean values $\pm$ SEM, **(B)** t-test: $p<0.001$ (=***), **(C)** 2-way ANOVA with Bonferroni post-test: ns = non-significant.

5.5 Role of 11β-HSD2 on Expression of Molecules involved in Stress Response

As the enzyme 11β-HSD2 regulates the availability of active cortisol inside of cells, downregulation of 11β-HSD2 might affect the stress response molecular machinery. In order to investigate such a potential deregulation, mRNA levels of CRH and its receptors, GR, MR, glucocorticoid- and mineralocorticoid-responsive genes as well as the transporter molecule BCRP were determined following siRNA knockdown of 11β-HSD2.

5.5.1 Role of 11β-HSD2 on Expression of CRH and its Receptors

Downregulation of 11β-HSD2 significantly increased CRH, CRH-R1 and CRH-R2 mRNA expression by 3.0-fold, 2.6-fold and 3.7-fold, respectively, after 24 h of siRNA treatment (Figure 5.13). Treatment for 48 h with siRNA showed a more variable and less potent result, but an upregulation of the three molecules was still evident after 11β-HSD2 depletion.

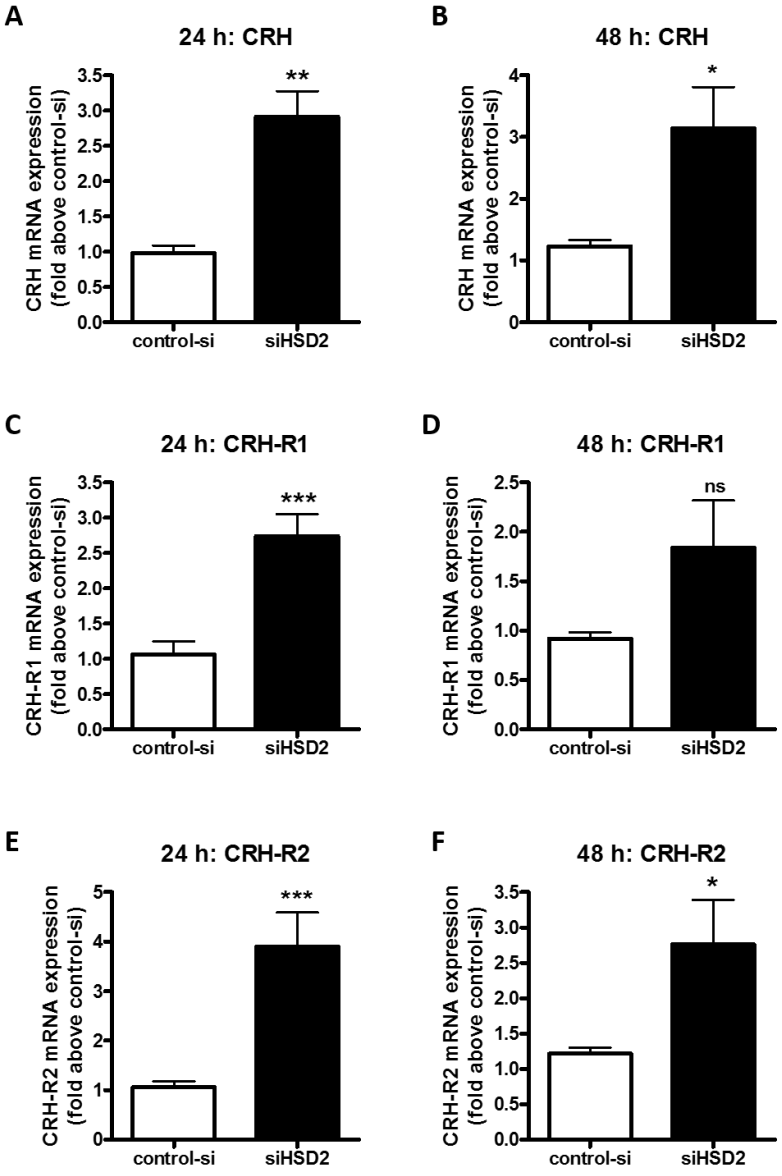

Figure 5.13: **Expression of CRH and its receptors in BeWo cells after 11β-HSD2 knockdown.** BeWo cells were treated for 24 h or 48 h with either control-siRNA or siHSD2 before qRT-PCR for CRH **(A, B)**, CRH-R1 **(C, D)** and CRH-R2 **(E, F)** mRNA was performed. qRT-PCR data was normalized to RPLP0 expression, n=4, data are expressed as mean values ± SEM, t-test: ns = non-significant, $p<0.05$ (=*), $p<0.01$ (=**), $p<0.001$ (=***).

The relationship between CRH, CRH-R1, CRH-R2 and 11β-HSD2 was also tested in differentiated BeWo cells (for treatment protocol see Figure 2.3 (1b) in chapter Material and Methods).

Figure 5.14 (A and C) show that the previously observed significant upregulation of CRH and CRH-R2 after siHSD2 treatment was still detectable in undifferentiated BeWo cells under an experimental setup that included treatment with 24 h siRNA with and without 100 μM forskolin. The expression of CRH-R1 showed a trend to an upregulation after 11β-HSD2 knockdown, although it failed to reach significance (Figure 5.14 B). Forskolin treatment led to upregulation of CRH and its receptors which is in agreement with the result from chapter 4.2.1 (Figure 4.11). Regarding CRH and CRH-R2, a combination of 11β-HSD2 knockdown and forskolin treatment led to the highest upregulation of CRH and CRH-R2 suggesting that forskolin effects were more potent in the absence of 11β-HSD2 (Figure 5.14 A and C).

I also investigated whether absence of 11β-HSD2 rendered the system more sensitive to the action of cortisol. The cortisol treatment abolished the siHSD2-induced upregulation of CRH and CRH-R2 in undifferentiated cells, whereas it did not have an effect in states of cAMP/PKA activation (induced by forskolin) (Figure 5.14 A). Regulation of CRH-R1 was neither influenced by 11β-HSD2 knockdown nor by cortisol (Figure 5.14 B), however in the presence of cortisol 11β-HSD2 knockdown led to an upregulation of CRH-R1 mRNA in differentiated cells (Figure 5.14 D).

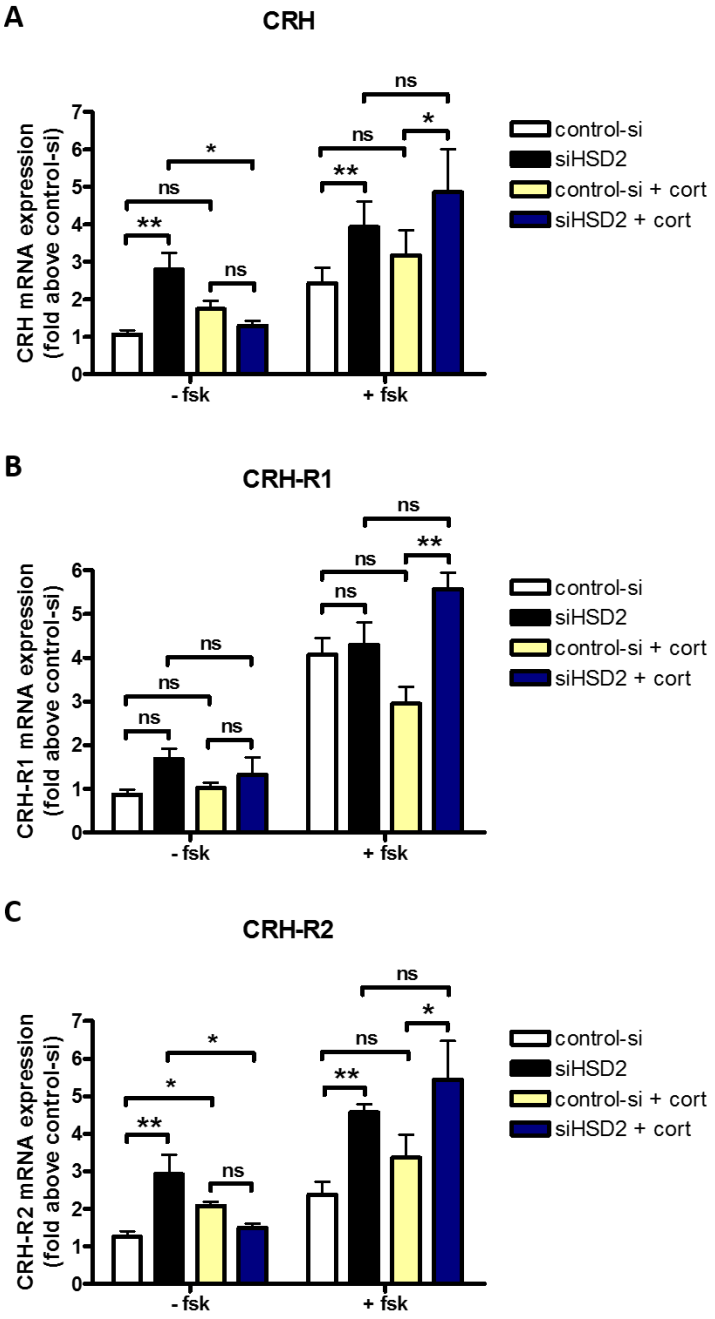

Figure 5.14: **Expression of CRH and its receptors after 11β-HSD2 knock-down ($\pm$ forskolin and cortisol treatment).** BeWo cells were treated for 24 h with either control-siRNA or siHSD2 and for another 24 h with 100 μM forskolin and 500 nM cortisol before qRT-PCR for CRH **(A)**, CRH-R1 **(B)** and CRH-R2 **(C)** was performed. qRT-PCR data was normalized to RPLP0 mRNA expression, n=4, data are expressed as mean values $\pm$ SEM, 2-way ANOVA with Bonferroni post test: ns = non-significant, $p<0.05$ (=*), $p<0.01$ (=**).

5.5.2 Role of 11β-HSD2 on Expression of GR, MR and its Target Genes

5.5.2.1 Role of 11β-HSD2 on Expression of GR, MR

I next investigated whether 11β-HSD2 played a role in regulating cortisol interactions with its intracellular receptors GR and MR. The mRNA level of GR and MR were determined in cells depleted of 11β-HSD2 and stimulated with either forskolin, cortisol or both. Figure 5.15 A shows that in undifferentiated cells neither knockdown of 11β-HSD2 nor cortisol treatment had a significant effect on GR mRNA expression. In differentiated cells, cortisol after 11β-HSD2 depletion led to a slight upregulation by 1.4-fold of GR mRNA compared to cells treated with cortisol in the presence of 11β-HSD2 (Figure 5.15 A), whereas all other treatments did not influence expression of GR.

Regarding MR mRNA expression, forskolin had an inhibitory effect by downregulating MR mRNA expression (Figure 5.15 B), as previously shown (Figure 4.13). In differentiated cells, cortisol after 11β-HSD2 depletion significantly, but slightly upregulated MR mRNA expression compared to cells treated with cortisol in the presence of 11β-HSD2 and compared to cells depleted of 11β-HSD2 (without cortisol treatment), whereas under all other experimental conditions no effect of siHSD2 was detectable. This result suggests that cortisol in the absence of 11β-HSD2 was able to modulate effects on GR and MR mRNA expression in differentiated cells.

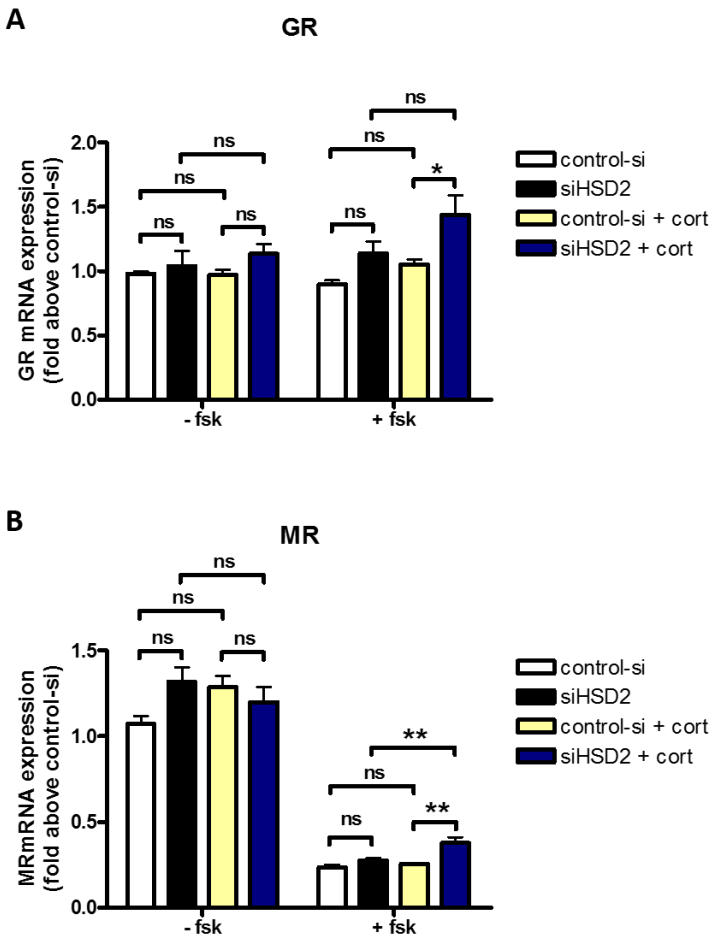

Figure 5.15: **Expression of GR and MR after 11β-HSD2 knockdown ($\pm$ forskolin and cortisol treatment).** BeWo cells were treated for 24 h with either control-siRNA or siHSD2 and for another 24 h $\pm$ 500 nM cortisol in the absence or presence of 100 μM forskolin before qRT-PCR for GR **(A)** and MR **(B)** was performed. qRT-PCR was normalized to 18S rRNA expression, n=4, data are expressed as mean values $\pm$ SEM, 2-way ANOVA with Bonferroni post test: ns = non-significant, $p<0.05$ (=*), $p<0.01$ (=**).

5.5.2.2 Role of 11β-HSD2 on Expression of Gluco- and Mineralocorticoid Target Genes

Since the enzyme 11β-HSD2 regulates the availability of active cortisol within the cells and ultimately cell responsiveness to cortisol, mRNA expression of two glucocorticoid-responsive genes, Dusp1 (dual specificity protein phosphatase 1) and Per1 (period circadian protein homolog 1), were investigated in BeWo cells after 11β-HSD2 knockdown and treatment with forskolin. Moreover, cells were stimulated with cortisol to determine the cell responsiveness to cortisol after 11β-HSD2 depletion. Figure 5.16 A shows that neither 11β-HSD2 knockdown nor cortisol had an effect on the mRNA expression of Dusp1 in un- and differentiated BeWo cells. Similarly, mRNA expression of Per1 was not affected by 11β-HSD2 knockdown nor by cortisol in differentiated cells (Figure 5.16 B). Only in undifferentiated cells, a slight 1.3-fold upregulation of Per1 mRNA after 11β-HSD2 knockdown was observed, this effect was abolished by simultaneous cortisol treatment. Since these genes are normally extremely sensitive to the action of cortisol, this minimal increase observed here raised concerns about its biological relevance. In contrast, treatment of BeWo cells with forskolin led to an up-regulation of both Dusp1 and Per1 mRNA in all experimental conditions. This forskolin-induced effect showed no synergistic action of cortisol nor by the 11β-HSD2 knockdown. This results suggests that regulation of glucocorticoid-responsive genes in the BeWo cells might be under the control of the cAMP pathway and might not be regulated by cortisol.

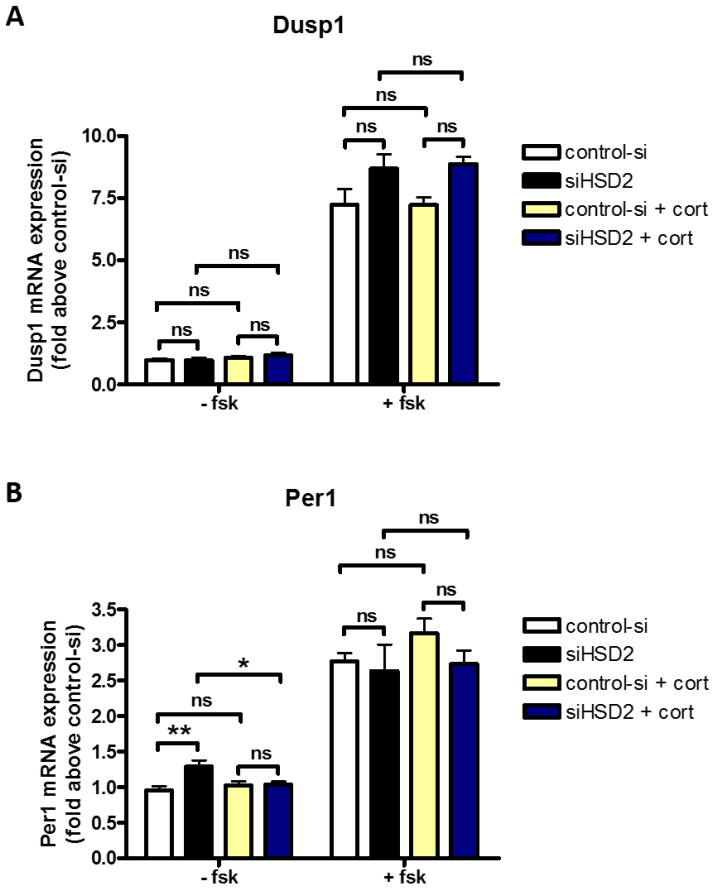

Figure 5.16: **Expression of Dusp1 and Per1 after 11β-HSD2 knockdown ($\pm$ forskolin and cortisol treatment).** BeWo cells were treated for 24 h with either control-siRNA or siHSD2 and another 24 h $\pm$ 500 nM cortisol in the absence or presence of 100 μM forskolin before qRT-PCR for Dusp1 **(A)** and Per1 **(B)** was performed. Dusp1 qRT-PCR was normalized to 18S rRNA and Per1 qRT-PCR to RLPL0 mRNA expression, n=4, data are expressed as mean values $\pm$ SEM, 2-way ANOVA with Bonferroni post test: ns = non-significant, $p<0.05$ (=*), $p<0.01$ (=**).

It might be possible that cortisol preferentially binds and activates MR rather than GR because of its higher affinity to the MR. Therefore, mRNA levels of SGK1 (serum- and glucocorticoid-regulated kinase 1) and ATP1A1 (ATPase subunit α 1), which are regulated by both GR and MR, were determined after 11β-HSD2 knock-down. Figure 5.17 A and B show that, similar to the regulation of the glucocorticoid-responsive genes (see above), the genes SGK1 and ATP1A1 were neither affected by siHSD2 nor cortisol, while forskolin treatment led to a significant upregulation of both molecules. Also this result suggests that regulation of mineralocorticoid-responsive genes in the BeWo cells might be under the control of the cAMP pathway and might not be regulated by cortisol.

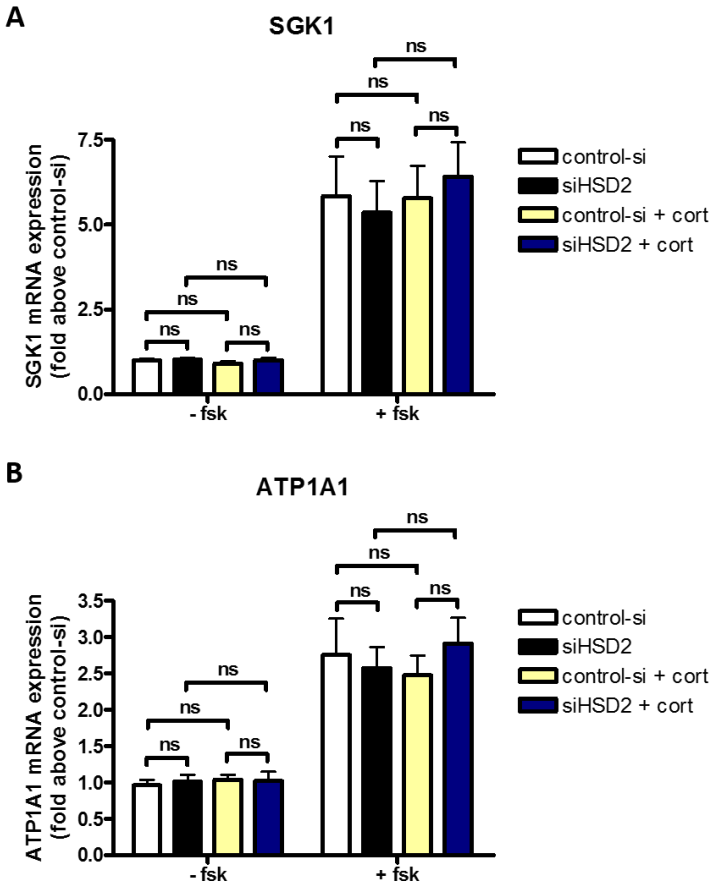

Figure 5.17: **Expression of SGK1 and ATP1A1 after 11β-HSD2 knockdown ($\pm$ forskolin and cortisol treatment).** BeWo cells were treated for 24 h with either control-siRNA or siHSD2 and another 24 h $\pm$ 500 nM cortisol in the absence or presence of 100 μM forskolin before qRT-PCR for SGK1 **(A)** and ATP1A1 **(B)** was performed. Normalization to 18S rRNA, n=4, data are expressed as mean values $\pm$ SEM, 2-way ANOVA with Bonferroni post test: ns = non-significant.

5.5.3 Role of 11β-HSD2 on Expression of BCRP

The transporter molecule BCRP was upregulated by forskolin (Figure 5.18) which was in agreement with the result from chapter 4.2.4 (Figure 4.17). Again, treatments with either siHSD2 or cortisol did not influence the mRNA expression of BCRP.

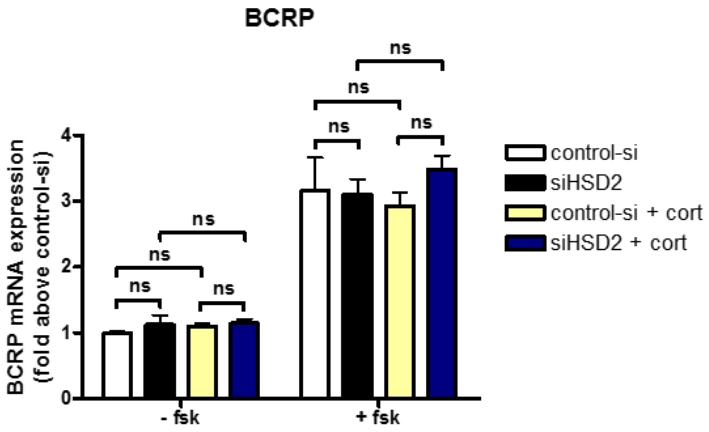

Figure 5.18: **Expression of BCRP after 11β-HSD2 knockdown ($\pm$ forskolin and cortisol treatment).** BeWo cells were treated for 24 h with either control-siRNA or siHSD2 and another 24 h $\pm$ 500 nM cortisol in the absence or presence of 100 μM forskolin before qRT-PCR for BCRP was performed. Normalization to 18S rRNA, n=4, data are expressed as mean values $\pm$ SEM, 2-way ANOVA with Bonferroni post test: ns = non-significant.

5.6 Regulation of 11β-HSD2 by CRH

Previous studies identified placental 11β-HSD2 as a target of pathological processes that are associated with adaptive responses and raised CRH. Therefore, the possible regulation of 11β-HSD2 by CRH was investigated.

In primary trophoblasts, it has been shown by Sharma et al. [2009] that 11β-HSD2 expression and activity is diminished by a p38 MAPK inhibitor and it has been shown by Guan et al. [2013] that 11β-HSD2 expression and activity is increased by an $ERK_{1/2}$ MAPK inhibitor. Therefore, the p38 MAPK inhibitor SB202190 and the $ERK_{1/2}$ MAPK inhibitor UO126 were used to confirm this in my experimental setup in addition to the PI3K (phosphatidyinositide 3 kinase) inhibitor Wortmannin to test whether the PI3K signalling pathway is also involved in the 11β-HSD2 regulation as well.

The experimental setup included a 24 h pre-treatment with the inhibitors (for treatment protocol see Figure 2.3 (3) in chapter Material and Methods). Subsequently, for measuring 11β-HSD2 mRNA levels, BeWo cells were incubated with and without 100 μM forskolin for 24 h in the presence of the inhibitors. For determining the 11β-HSD2 activity by measuring the remaining cortisol concentration in the supernatant, BeWo cells were incubated with and without 100 μM forskolin for 24 h in the presence of 500 nM cortisol and the inhibitors.

Inhibition of p38 activity with the inhibitor SB202190 led to a significant decrease of 11β-HSD2 mRNA expression by 87% in undifferentiated BeWo cells compared to control-treated cells (Figure 5.19 A). Also, in differentiated BeWo cells (with forskolin treatment), the SB202190 inhibitor significantly attenuated the forskolin-induced upregulation of 11β-HSD2 mRNA, albeit to a lesser extent (45%). The 11β-HSD2 activity assay showed that treatment with SB202190 compared to control samples (Figure 5.19 B) decreased 11β-HSD2 activity (= increased remaining cortisol in the supernatant) in un- and differentiated BeWo cells which is consistent with the lower mRNA expression of 11β-HSD2 after inhibition of the p38 MAP-kinase.

In agreement with published data, use of the UO126 inhibitor significantly increased the 11β-HSD2 mRNA in un- and differentiated BeWo cells and enhanced enzymatic activity as shown by a significantly lower concentration of remaining cortisol in the supernatant compared with its control samples (Figures 5.19 C and D).

These data confirm previous reports that p38 and $ERK_{1/2}$ MAPKs exert opposing effects on 11β-HSD2 mRNA expression and activity in primary trophoblast

cells as it was shown by Sharma et al. [2009] and Guan et al. [2013], respectively.

Inhibition of PI3-kinase by Wortmannin did not alter 11β-HSD2 mRNA expression nor activity in un- and differentiated BeWo cells, although a trend to a higher mRNA expression and activity of 11β-HSD2 was observed (Figures 5.19 E and F).

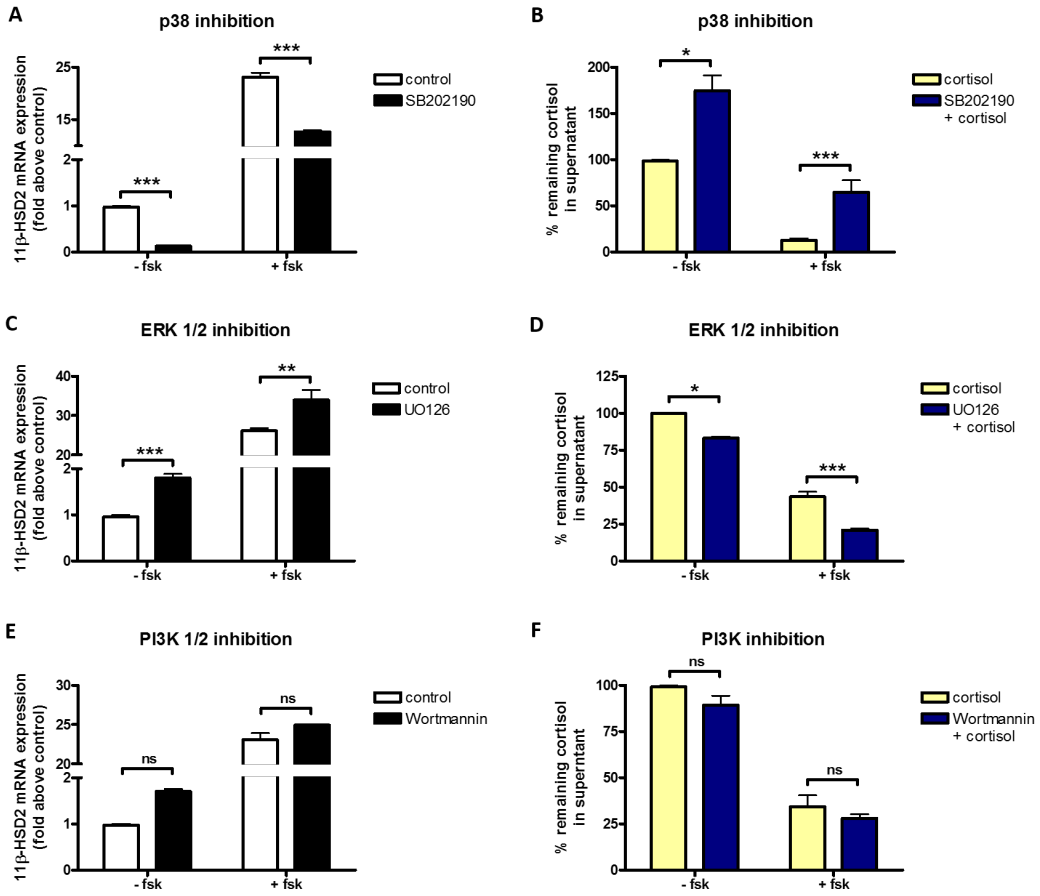

Figure 5.19: **Signalling pathways involved in the regulation of 11β-HSD2.** BeWo cells were pre-treated for 24 h with **(A, B)** p38 inhibitor SB202190, **(C, D)** ERK1/2 inhibitor UO126 and **(E, F)** PI3K inhibitor Wortmannin. After pre-treatment, the cells were treated with the inhibitors for further 24 h with or without 100 μM forskolin for qRT-PCR experiments (in the absence of cortisol) **(A, C, E)** or in the presence of 1 μM cortisol for measuring the cortisol concentration in the supernatant **(B, D, F)**. **(A, C, E)** Normalization to 18S rRNA, **(B, D, F)** normalization to protein concentration, n=3, data are expressed as mean values $\pm$ SEM, 2-way ANOVA with Bonferroni post test: ns = non-significant, $p<0.05$ (=*), $p<0.01$ (=**), $p<0.001$ (=***).

Since previous studies showed that CRH activates both p38 and $ERK_{1/2}$ MAPK signalling cascades, I next investigated a potential regulation of 11β-HSD2 by CRH. BeWo cells were treated with different concentrations of CRH with and without forskolin in the absence (to measure 11β-HSD2 mRNA expression) or presence of cortisol (to estimate 11β-HSD2 activity). High CRH concentration (1 μM CRH) led to a small, but significant, increase in 11β-HSD2 mRNA in differentiated BeWo cells (Figure 5.20). However, 11β-HSD2 activity did not seem to be affected by this effect, as no difference in remaining cortisol concentration in the supernatant in this condition could be detected. This result suggests that CRH does not regulate the activity of 11β-HSD2.

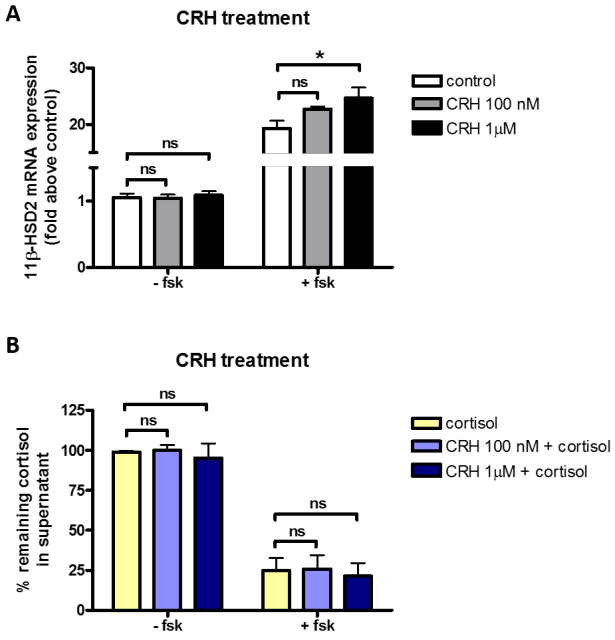

Figure 5.20: **CRH effect on 11β-HSD2 activity.** BeWo cells were pre-treated for 24 h with 100 nM or 1 μM CRH. After pre-treatment, the cells were treated with CRH for further 24 h with or without 100 μM forskolin for qRT-PCR experiments (in the absence of cortisol) **(A)** or in the presence of 1 μM cortisol for measuring the cortisol concentration in the supernatant **(B)**. **(A)** Normalization to 18S rRNA, **(B)** normalization to protein concentration, n=3, data are expressed as mean values $\pm$ SEM, 2-way ANOVA with Bonferroni post test: ns = non-significant, $p<0.05$ (=*).

5.7 Discussion of Chapter 5

The data of this chapter show that the enzyme 11β-HSD2 plays key roles in essential BeWo cell biology processes that are not directly associated with cortisol action (Figure 5.21 for summary). Cell differentiation is positively regulated by 11β-HSD2 since knockdown of 11β-HSD2 limited the differentiation effect, both on the cell fusion process as well as on the biochemical differentiation of BeWo cells. Moreover, 11β-HSD2 seemed to enhance BeWo cell viability and protected against apoptosis as knockdown of the enzyme led to a transient decrease in cell viability associated with increased apoptosis. A critical role of 11β-HSD2 in the regulation of placental hormones such as CRH was also demonstrated in the BeWo cells. Expression of CRH and selectively its receptor CRH-R2 was limited by 11β-HSD2 which might prevent an overactivation of the CRH-cortisol positive feedback loop in the placenta. Furthermore, neither 11β-HSD2 nor cortisol were involved in the expression of gluco- and mineralocorticoid target genes, but instead cAMP signalling might control the regulation of these genes in BeWo cells.

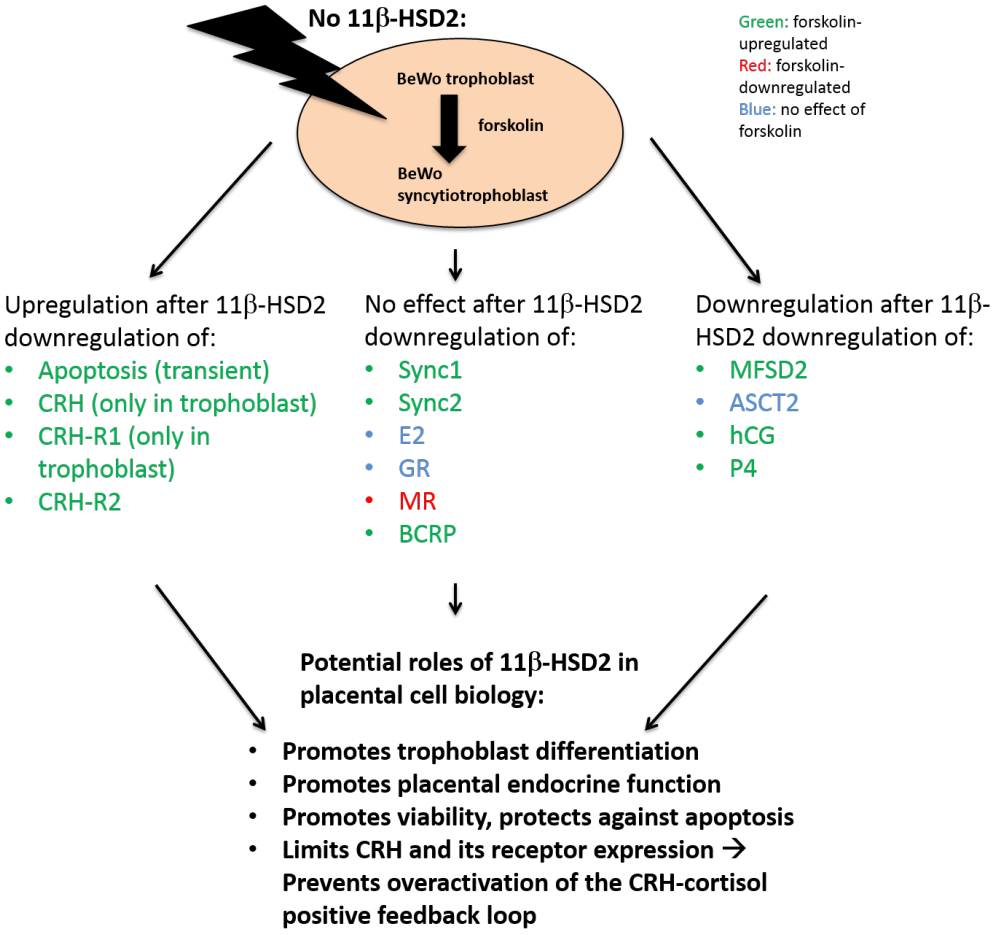

Figure 5.21: **Summary of effects after 11β-HSD2 downregulation.**

The fusogenes Syncytin-1 and -2 and their receptors are involved in the fusion process of trophoblast cells. Upregulation of Syncytin-1 and -2 mRNA after forskolin treatment in BeWo cells is well established (Chapter 4.1.2) and was confirmed in the experiments of this chapter. In differentiated BeWo cells, both Syncytin receptors ASCT2 and MFSD2 were positively regulated by 11β-HSD2. A reduced expression of Syncytin receptors might diminish activation of signalling pathways which trigger fusion of BeWo cells even though enough ligand is available. This result might suggest a pro-differentiating role of 11β-HSD2. Possibly, the potential alterations in the stimulation of the Syncytin receptors might be associated with activation of apoptosis as Knerr et al. [2007] and Knerr et al. [2008] showed that overexpression of Syncytin-1 in HEK 293 and CHO attenuated STS- and antimycin A-induced apoptosis.

Downregulation of 11β-HSD2 in BeWo cells led to a decreased secretion of hCG and progesterone in un- and differentiated cells suggesting a role of 11β-HSD2 in promoting placental endocrine function and hormone secretion. Cortisol treatment did not display any effect on either hCG or progesterone secretion suggesting that 11β-HSD2 might have additional roles besides its enzymatic inactivation of cortisol. Because secretion of estradiol was not reduced after 11β-HSD2 knockdown, the reduced secretion of hCG and progesterone appears to be a selective effect.

Comparing these data with results from the other relevant studies, it seems that there is an interplay between 11β-HSD2 and hCG. Ni et al. [2009] and Shu et al. [2014] demonstrated that treatment of syncytiotrophoblast with exogenous hCG leads to an upregulation of 11β-HSD2 expression. However, in contrast to my experiments they investigated the effect of hCG on expression of 11β-HSD2. Ni et al. [2009] used an hCG antibody to neutralize hCG secretion which leads to a reduced expression of 11β-HSD2 in primary syncytiotrophoblast. In addition, treatment of the primary syncytiotrophoblast with exogenous hCG increases 11β-HSD2 expression. Shu et al. [2014] further explored the hCG effect on upregulating 11β-HSD2 mRNA and protein expression and could demonstrate that p38 signalling is involved in this process. As these two studies were different in the experimental setup, a direct comparison with my results is not appropriate. Nevertheless, it is noteworthy that there might be feedback mechanism between 11β-HSD2 and hCG maintaining the expression of both molecules.

As mentioned previously, hCG promotes fusion of trophoblast cells [Dhar et al., 2004]. Thus, it might be possible that the above mentioned role of 11β-HSD2 in promoting BeWo cell fusion might be associated with hCG secretion.

Sun et al. [1998] identified an inhibitory effect of progesterone on 11β-HSD2 mRNA expression and activity in primary syncytiotrophoblast. Again, this experimental setup is substantially different compared to my study. Jeschke et al. [2005] and Yang et al. [2006] showed that CRH reduces progesterone secretion in primary syncytiotrophoblasts and Gao et al. [2012] showed that inhibition of CRH or its receptor leads to an increased progesterone production. In the BeWo cell model, the 11β-HSD2 knockdown led to an increase in CRH mRNA expression in un- and differentiated BeWo cells suggesting the possibility that this increased expression of CRH reduces P4 secretion.

To highlight the importance of these findings, both hormones hCG and progesterone fulfill various roles in the placenta in order to maintain a successful pregnancy (Chapter 1.2.7.2). For example, both hormones are involved in maintaining uterine quiescence [Eta et al., 1994; Ruddock et al., 2008]. Deregulation of hCG and P4 hormones could have adverse effects on the development of the fetus with a possible risk of pre-term labour. In several placental diseases, the expression and activity of the enzyme 11β-HSD2 is reduced [Causevic and Mohaupt, 2007; Dy et al., 2008] and this might lead to an abnormal hormonal milieu of the placenta. Based on the BeWo cell results above, the reduced 11β-HSD2 expression could potentially alter the endocrine function of the placenta *in vivo*.

Regarding cell turnover of BeWo cells, 11β-HSD2 seems to promote cell viability and protects against apoptosis. Reduced viability might be a result of decreased proliferation or increased apoptotic or necrotic processes. Theoretically, the expression of 11β-HSD2 might have a pro-proliferation effect as it inactivates the release of anti-proliferative cortisol. In other cellular models, Rabbitt et al. [2002] transfected 11β-HSD2 in rat osteosarcoma cells and observed a pro-proliferative effect. Koyama and Krozowski [2001] showed that use of glycyrrhetinic acid (inhibitor of the 11β-HSD enzymes) in Ishikawa endometrial cancer cells (express only 11β-HSD2 and no 11β-HSD1) decreases the cell number as determined by counting cells after trypsinization. My result of a reduction in BeWo cell number (possibly because of less proliferation) after 11β-HSD2 knockdown would be in agreement with the observation by Koyama and Krozowski [2001]. Another explanation of the lower cell number after 11β-HSD2 knockdown might also be related to decreased hCG production. As discussed, downregulation of 11β-HSD2 led to a fall in hCG secretion in BeWo cells. Hamada et al. [2005] showed that downregulation of hCG in JAR cells leads to a decreased proliferation.

The modulation of apoptosis by 11β-HSD2 is often observed in tumor cells. Several groups showed that inactivation of 11β-HSD2 with simultaneous treatment with cortisol provokes apoptosis in different cell types. Tao et al. [2013] showed that Jurkat leukemia T cells display more apoptosis after cortisol treatment (Annexin V/PI assay) when 11β-HSD2 was inactivated with an inhibitor as well as with a 11β-HSD2 shRNA. Inhibition of 11β-HSD2 with carbenoxolone in MOLT4F cells leads to more apoptotic cells after prednisolone treatment as assessed by caspase-3/7 assay [Sai et al., 2011]. Also, carbenoxolone used in murine corticotroph tumor cells induces apoptosis after cortisol treatment demonstrated with a TUNEL-staining [Nigawara et al., 2006]. In agreement with these observation, knockdown of 11β-HSD2 for 24 h led to an increased activation of caspase-3/7 in BeWo cells. Demonstrating the same effect with a different approach, O'Brien et al. [2004] transfected cell-specifically osteoblast/osteocyte cells in an *in vivo* mice model with an 11β-HSD2 expressing vector which protects the cells against glucocorticoid-induced apoptosis.

Interestingly, during differentiation of trophoblast cells, the apoptotic cascade is activated (Chapter 1.2.6.3). Therefore, upregulation of 11β-HSD2 in the syncytiotrophoblast might be a mechanism of the placenta to control and protect the developing syncytiotrophoblast against pursuing the whole apoptotic process.

As discussed above, knockdown of 11β-HSD2 led to a decrease in hCG and progesterone production. It was shown by Jasinska et al. [2006], Kajihara et al. [2011a] and Kajihara et al. [2011b] that hCG treatment leads to an increased expression of the anti-apoptotic Bcl-2 in endometrial stromal cells. Furthermore, Liu et al. [2007] showed that progesterone treatment in HTR-8/SV cells (model system of extravillous trophoblast cells) leads to less apoptosis characterized by decreased Fas, FasL, caspase-8, caspase-3 and PARP expression and by increased Bcl-2 expression. Similarly to that, it might be possible that hCG and progesterone are involved in regulating apoptosis in BeWo cells and that 11β-HSD2 protects against apoptosis via a mechanism involving the sufficient expression of hCG and progesterone.

Taken together, my result revealed a role of 11β-HSD2 in protecting against apoptosis in trophoblast cells as it has been shown before in other cell types.

My experiments identified an interesting relationship between 11β-HSD2 and the molecular machinery controlling CRH expression. In undifferentiated BeWo cells, downregulation of 11β-HSD2 led to a significant increase in CRH and both its receptor (CRH-R1 and CRH-R2) mRNA after 24 h (Figure 5.13). This result suggests that in the trophoblast 11β-HSD2 might limit the CRH production and is thereby potentially preventing an overactivation of the cortisol-CRH positive feed-

back loop. In the placenta, cortisol stimulates CRH expression [Robinson et al., 1988] resulting in a positive feedback loop. Moreover, it has been shown that cortisol leads to an increase of placental CRH expression, whereas progesterone had the opposite effect [Jones et al., 1989]. However, the positive effect of cortisol on placental CRH expression requires simultaneous presence of progesterone since Karalis et al. [1996] could show in a primary trophoblast model that cortisol also had an inhibitory effect on CRH mRNA expression when the cells were cultured in serum-free media which guaranteed a progesterone-free milieu. In further experiments, they revealed that progesterone inhibits CRH expression to a greater extend than cortisol. According to this, cortisol and progesterone compete for binding to the GR (progesterone binds to the GR [Ojasoo et al., 1988]) and higher levels of cortisol block progesterone binding to the GR and consequently block the inhibitory effect of progesterone on CRH expression [Karalis and Majzoub, 1995]. As 11β-HSD2 inactivates cortisol, more progesterone could bind to the GR due to less competition with active cortisol and as a consequence reduced levels of CRH are produced. Depletion of 11β-HSD2 by siHSD2 might result in more cortisol available in the BeWo cells for binding to GR leading to a higher CRH production as shown in my experiments in undifferentiated BeWo cells (only treated with siHSD2).

The regulation of CRH and CRH-R2 seems to be similar regarding effects of 11β-HSD2 knockdown and forskolin and cortisol treatment. In the absence of cortisol, siHSD2 and forskolin display effects on CRH and CRH-R2 mRNA expression in a synergistic manner. When performing the same experiment in the presence of cortisol, the siHSD2-induced upregulation of CRH and CRH-R2 mRNA expression was abrogated in undifferentiated BeWo cells. This result is surprising because glucocorticoids upregulate CRH in the placenta [Jones et al., 1989]. A combination of exogenous cortisol and knockdown of 11β-HSD2 would be expected to lead to the highest CRH mRNA expression because all of the exogenous cortisol could exert its action and thereby lead to upregulation of CRH. As this was not the case, the result suggests that the siHSD2-induced upregulation of CRH and CRH-R2 mRNA (in the absence of cortisol) is independent of the role of 11β-HSD2 to inactivate cortisol.

As BeWo cells might be quite insensitive to glucocorticoids as shown before [Mark and Waddell, 2006], the responsiveness of our BeWo cells to cortisol after 11β-HSD2 knockdown was tested. Neither siHSD2 nor cortisol treatment had an effect on the gluco- and mineralocorticoid responsive genes Dusp1, Per1, SGK1, and ATP1A1. However, forskolin treatment led to an mRNA upregulation of all of these genes suggesting that the cAMP signalling pathway might be involved in the GC

regulation of gluco- and mineralocorticoid-responsive genes in BeWo cells. In order to investigate this phenomenom further, several experiments have been performed which are presented in chapter 6.

This insensitivity to cortisol strengthens the conclusion that the observable effects of 11β-HSD2 downregulation in BeWo cells regarding differentiation, endocrine function, viability, apoptosis, and stress regulation is independent of cortisol and suggests that the enzyme 11β-HSD2 might fulfill roles other than just regulation of cortisol activity.

Chapter 6

Glucocorticoid Action via GR and its Effects on BeWo Cell Biology

My previous results provided strong, albeit indirect, support for a lack of glucocorticoid effects in BeWo cells. To characterize this further, the responsiveness of BeWo cells to glucocorticoids was tested using a reporter-gene-assay approach as well as expression studies of glucocorticoid-responsive genes. Additionally, the involvement of cAMP signalling in glucocorticoid signalling was also investigated.

Moreover, glucocorticoid effects on BeWo cell turnover, differentiation and expression of molecules involved in the stress response and glucocorticoid action molecular machinery was investigated. This was driven by the fact that in several *in vivo* conditions such as psychological stress or chronic diseases, e.g. asthma, which are treated with glucocorticoids, excess of glucocorticoids reach placental tissue. The enzyme 11β-HSD2 controls the cellular amount of active cortisol available for receptor activation within the tissue and its reduced expression of 11β-HSD2 in placental diseases such pre-eclampsia and IUGR might lead to increased amounts of active cortisol. Elevated levels of cortisol can pass through the placenta to induce effects in the fetus, but it is also possible that active cortisol binds to the placental gluco- or mineralocorticoid receptor leading to an altered glucocorticoid action within the placenta. Taken together, several physiological and pathological situations might cause increased levels of cortisol/glucocorticoids reaching the placental tissue, which highlights the importance to investigate this mechanism.

6.1 Comparison of GRα Expression in BeWo and HeLa Cells

It was previously shown that BeWo cells express GRα mRNA whose expression was not affected by forskolin treatment or by 11β-HSD2 depletion (Figure 4.13 and 5.15).

The expression of GRα mRNA in BeWo cells was compared with HeLa cells which are highly responsive to glucocorticoids [Melnykovych and Bishop, 1969, 1971]. Using qRT-PCR my results showed that HeLa cells expressed 10.7-fold more GRα than BeWo cells (Figure 6.1 A) which might be a reason why BeWo cells are less or non-responsive to glucocorticoids.

Furthermore, the feedback regulation of GRα by dexamethasone (a highly potent synthetic glucocorticoid) and/or forskolin was determined. HeLa cells down-regulated their GRα expression after dexamethasone (=Dex) treatment by 41-42% (in the presence and absence of forskolin) indicating the presence of a negative feedback mechanism (Figure 6.1 B). Forskolin showed no effects on GRα mRNA expression in HeLa cells. In contrast, GRα mRNA expression in BeWo cells was not affected by Dex or by forskolin treatment (Figure 6.1 C) suggesting the absence of any GR feedback mechanisms in BeWo cells.

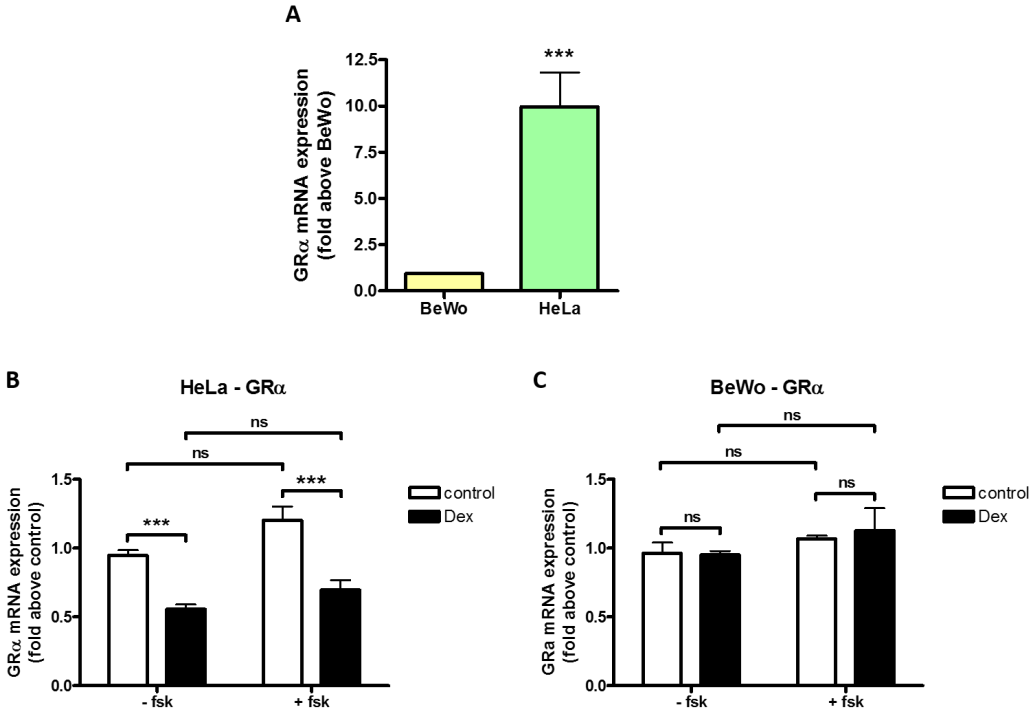

Figure 6.1: **mRNA expression of GRα in BeWo and HeLa cells. (A)** mRNA was extracted from BeWo and HeLa cells after 3 days of basal culture and a qRT-PCR for GRα was performed. **(B)** HeLa and **(C)** BeWo cells were treated with 10^{-6}M dexamethasone (=Dex) and/or 100 μM forskolin (=fsk) for 24 h before extracting mRNA and performing qRT-PCR for GRα. Normalisation to 18S rRNA, n=4, data are expressed as mean values $\pm$ SEM, fold above control = fold above control cells which were not treated with Dex nor fsk, ns = non-significant, $p<0.001$ (=***), **(A)** t-test, **(B, C)** 2-way ANOVA with Bonferroni post-test.

GRα Expression in BeWo Cells after Transfection with a GRα-vector

In order to adjust the low expression of GRα in BeWo cells, the human GRα gene expressed in a suitable vector (pRShGRα-vector, see chapter 2.2.2.2 for details) was transfected. Transfection efficiency was confirmed by qRT-PCR in BeWo cells. Figure 6.2 shows that the transfection of GRα led to a 736-fold higher GRα mRNA expression compared with the non-transfected BeWo cells.

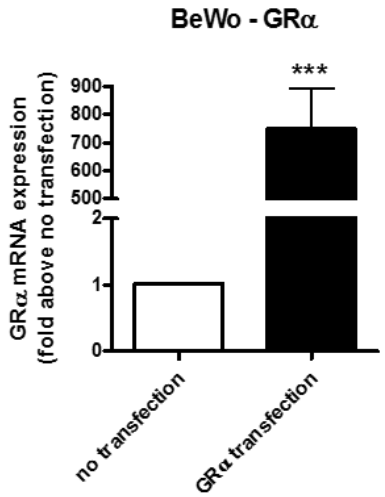

Figure 6.2: **mRNA expression of GRα in BeWo cells after GRα transfection.** BeWo cells were transfected with 2 μg GRα (pRShGRα-vector) for 4 h, mRNA was extracted 24 h after transfection and a qRT-PCR for GRα was performed. Normalisation to 18S rRNA, n=4, data are expressed as mean values $\pm$ SEM, $p<0.001$ (=***), t-test.

6.2 Effects of Glucocorticoids on GRE-dependent Luciferase Transcription

Following overexpression of GRα in BeWo cells, I investigated potential changes in cell responsiveness to glucocorticoids. To quantify this, a reporter-gene-assay was used which consisted of a GRE-containing luciferase DNA-vector (= MMTV-luc, (Chapter 2.2.2.2)). As a control cell line, HeLa cells which are highly responsive to glucocorticoids [Melnykovych and Bishop, 1969, 1971] were used. Furthermore, HEK 293 cells were also tested for their glucocorticoid response since they only express low amounts of GR (personal communication with Dr. Kino).

6.2.1 DNA-vector Transfection in BeWo, HEK 293 and HeLa Cells

A GFP-containing DNA-vector was transfected into BeWo, HEK 293 and HeLa cells to determine their transfection efficiency characteristics. Figure 6.3 shows that all cell lines could be transfected with the GFP-DNA-vector. HeLa cells seem to display the highest transfection efficiency compared to HEK 293 and BeWo cells.

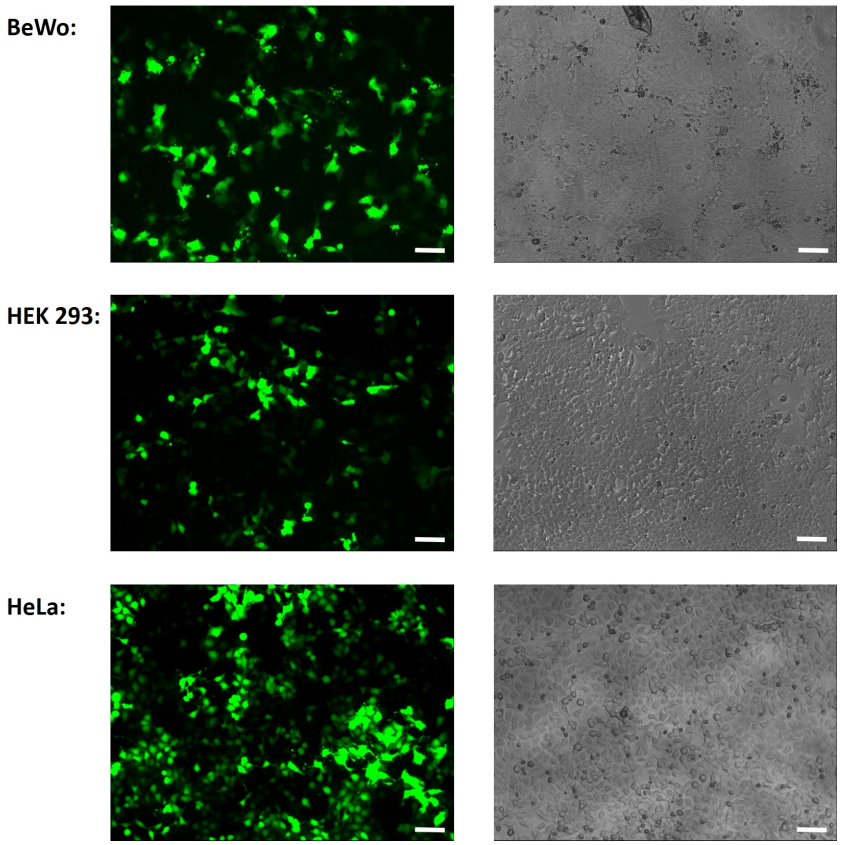

Figure 6.3: **GFP-vector transfection in BeWo, HEK 293 and HeLa cells.** BeWo, HeLa and HEK 293 cells were transfected with 2 μg of a GFP-containing DNA-vector for 24 h before fluorescent and brightfield microscopy was performed. Left: GFP (= green), right: brightfield. Representative images are shown, n = 3, bar = 75 μm.

6.2.2 GC Responsiveness of BeWo, HEK 293 and HeLa Cells

Glucocorticoids cross the cell membrane due to their lipophilicity and in the cytoplasm they bind to the GRα receptor, which homodimerizes and translocates to the nucleus where it acts as a transcription factor (Chapter 1.1.3.1). Most of the target genes have glucocorticoid responsive elements (GREs) in their promoter region that enable GR binding, which leads to either up- or downregulation of target genes [Tsai et al., 1988; Wrange et al., 1989].

To test responsiveness of BeWo cells to glucocorticoids, we transfected BeWo cells as well as HeLa and HEK 293 cells (as control cell lines) with the MMTV-luc vector containing GRE elements and encoding the *firefly*-luciferase with and without co-transfection of GRα (pRShGRα-vector) and stimulated them with 1 μM dexamethasone before measuring signal generation (for treatment protocol see Figure 2.3 (2) in chapter Material and Methods, Chapter 2.2.2.2 for experimental details).

HeLa cells expressed a functional GR as they showed a significant response to dexamethasone (Dex) after MMTV-luc transfection (190-fold) (Figure 6.4 A). Co-transfection of GRα and MMTV-luc led to a higher response to Dex (434-fold) compared to control cells (Figure 6.4 B).

HEK 293 cells also expressed a functional GR, but cell responsiveness was considerably lower after dexamethasone treatment (4.8-fold) compared to HeLa cells (Figure 6.4 C). Co-transfection with the GRα-vector increased the responsiveness to dexamethasone to 705-fold when comparing with control cells (Figure 6.4 D).

BeWo cells did not respond to dexamethasone in the absence of the exogenous GRα-vector (Figure 6.4 E). However, co-transfection of the GRα-vector restored the responsiveness to dexamethasone. BeWo cells transfected with the MMTV-luc and the GRα vectors showed a 322-fold induction of luciferase activity after Dex treatment compared to control cells suggesting that transfection of exogenous GRα restored glucocorticoid sensitivity.

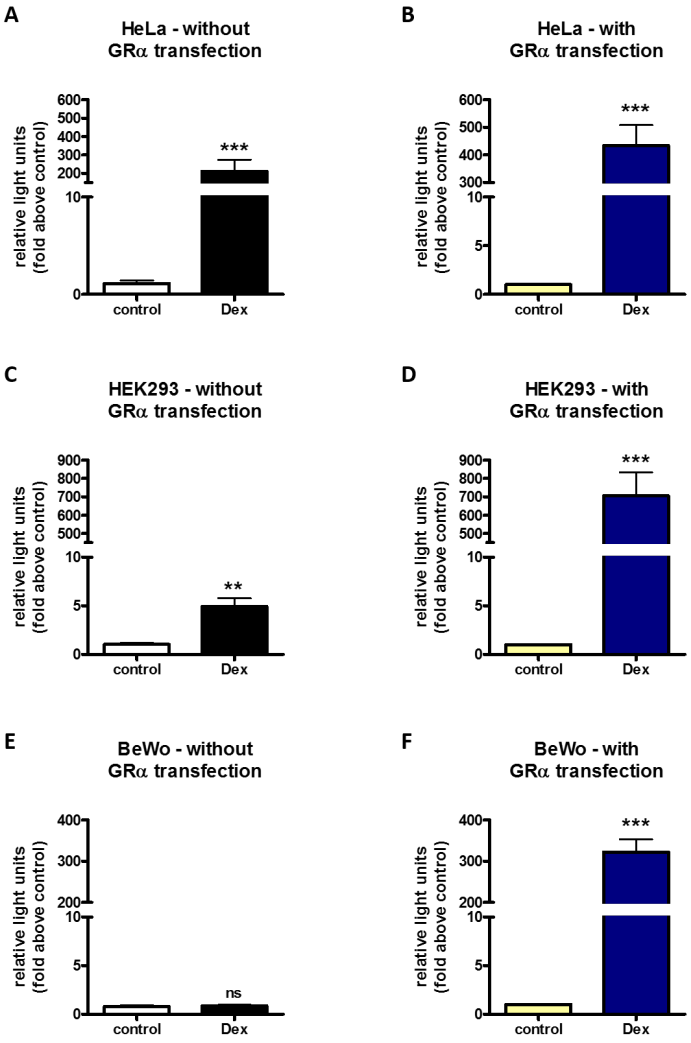

Figure 6.4: **HeLa, HEK 293, BeWo cell responsiveness to dexamethasone.** HeLa **(A, B)**, HEK 293 **(C, D)**, and BeWo cells **(E, F)** were co-transfected with the MMTV-luc- and pGL4.73-Rluc-vector with **(B, D, F)** and without **(A, C, E)** pRShGRα-vector for 4 h and subsequently treated with 10^{-6}M dexamethasone (=Dex) for 20 h before relative light units were determined. **(A, B)** n=4, **(C-F)** n=3, samples were normalized to *renilla*-luciferase activity, data are expressed as mean values ± SEM, ns = non-significant, p<0.01 (=**), p<0.001 (=***), t-test.

6.3 Effects of Glucocorticoids and Forskolin on Steroid-dependent Transcription

6.3.1 Effects of Dexamethasone and Forskolin on GC-responsive Genes via GR

Previous experiments raised the strong possibility that BeWo cells do not express endogenous functional GR, and therefore transfection of the GRα expression vector was introduced into the experimental protocol in order to determine the effects of glucocorticoids and forskolin on steroid-dependent transcription. My previous results suggested that in BeWo cells forskolin and the cAMP pathway was driving regulation of glucocorticoid-responsive genes in BeWo cells. These experiments used native BeWo cells without transfection of exogenous GRα and the less potent naturally occuring glucocorticoid cortisol compared with the highly potent synthetic glucocorticoid dexamethasone. To further test this experimental system, BeWo cells were treated with dexamethasone with and without co-treatment of forskolin in the absence and presence of exogenous GRα and either mRNA was extracted to perform qRT-PCRs for glucocorticoid-responsive genes or the cells were co-transfected with the MMTV-luc vector to perform reporter-gene-assays.

6.3.1.1 Effects of Dexamethasone and Forskolin on GC-responsive Genes in HeLa Cells

HeLa cells were used as a control cell line because of their high responsiveness to glucocorticoids to confirm glucocorticoid-induced upregulation of the specific glucocorticoid-responsive genes (Dusp1, Per1, Gilz). According to my experimental protocol, HeLa cells were treated with dexamethasone and/or forskolin for 24 h before determining mRNA expression levels of Dusp1, Per1 and Gilz.

Dexamethasone treatment led to a significant increase of Dusp1, Per1, and Gilz mRNA by 5.2-, 6.7-, and 14.7-fold, respectively, compared to control cells (Figure 6.5 A, B, C). Forskolin treatment significantly induced Dusp1 and Gilz mRNA expression by 2.1- and 1.8-fold (Figure 6.5 A, C), respectively, whereas mRNA expression of Per1 was not affected by forskolin treatment (Figure 6.5 B). Co-treatment of dexamethasone and forskolin did not change Per1 mRNA level compared to Dex treatment alone (Figure 6.5 B), whereas it significantly upregulated Dusp1 and Gilz mRNA expression by a furter 30% and 24%, respectively, compared to dexamethasone treatment alone (Figure 6.5 A, C). This result confirms responsiveness of HeLa

cells to glucocorticoids and suggests that some glucocorticoid-responsive genes are synergistically regulated by dexamethasone and forskolin.

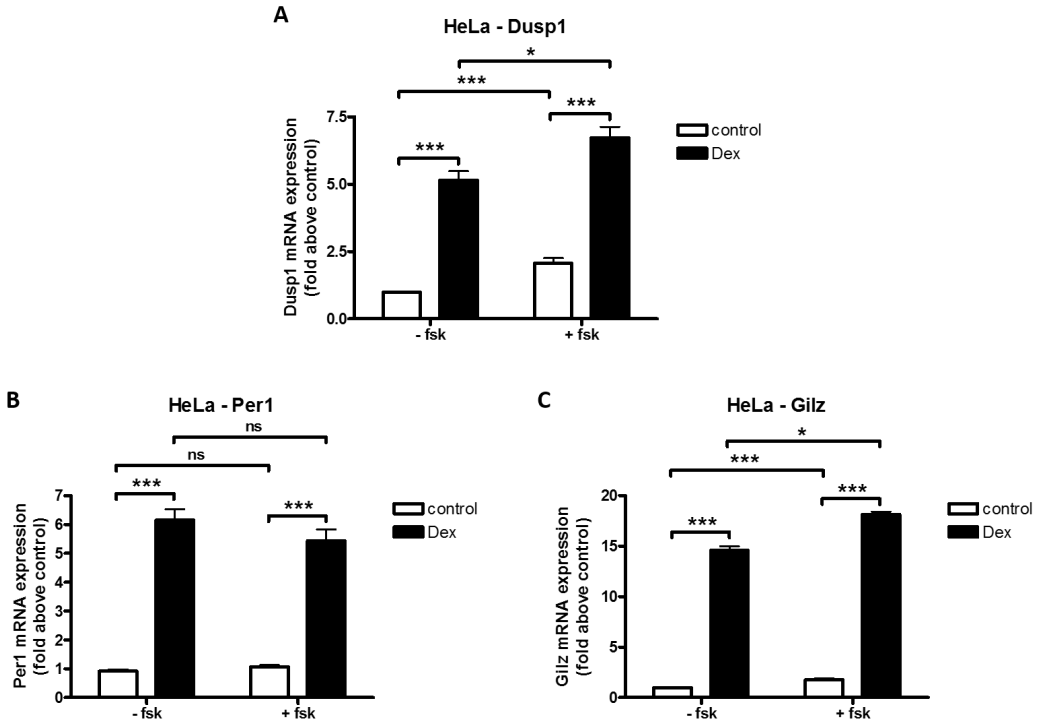

Figure 6.5: **Effect of dexamethasone and forskolin on GC-responsive gene expression in HeLa cells.** HeLa cells were treated with 10^{-6}M dexamethasone and/or 100 μM forskolin for 24 h before extracting the mRNA and performing qRT-PCR for Dusp1 **(A)**, Per1 **(B)**, and Gilz **(C)**. **(A)** Normalisation to 18S rRNA, **(B, C)** normalisation to GAPDH mRNA, n=4, data are expressed as mean values ± SEM, $p<0.05$ (=*), $p<0.001$ (=***), ns=non-significant, 2-way ANOVA with Bonferroni post-test.

6.3.1.2 Effects of Dexamethasone and Forskolin on GC-responsive Genes in BeWo Cells

Native and GRα-overexpressing BeWo cells were treated with dexamethasone and/or forskolin for 24 h before determining the mRNA expression levels of glucocorticoid-responsive genes. In native BeWo cells, dexamethasone did not have an effect on the mRNA expression of Dusp1, Per1 nor Gilz (Figure 6.6 A, C, E). Forskolin significantly increased the mRNA expression of Dusp1, Per1, and Gilz by 5.6-, 2.1-, and 1.8-fold, respectively, compared to control cells (Figure 6.6 A, C, E).

In BeWo-GRα cells (= BeWo cells overexpressing GRα), dexamethasone significantly increased the mRNA expression of Dusp1, Per1, and Gilz by 1.9-, 1.5-, and 83.5-fold, respectively, compared to control cells (Figure 6.6 B, D, F). Forskolin led to a significant increase of Dusp1 and Per1 by 3.7- and 2.2-fold, respectively, (Figure 6.6 B, D) which is comparable to the situation in native BeWo cells. Co-treatment of dexamethasone and forskolin in BeWo-GRα cells led to the highest induction of Dusp1 and Per1 by 5.9- and 3.3-, respectively, (Figure 6.6 B, D) indicating synergistic pathways. In case of Gilz, forskolin treatment did not affect the Gilz mRNA level, nor did it further increase the dexamethasone-induced Gilz mRNA upregulation which suggests that Gilz is exclusively regulated through glucocorticoid-induced GRα pathways (Figure 6.6 F).

This result suggests that in the absence of exogenous GRα in BeWo cells, forskolin regulates GC-responsive genes. In the presence of functional exogenous GRα, Dusp1 and Per1 are regulated by dexamethasone and forskolin synergistically, whereas Gilz appears to be strongly regulated by glucocorticoids only.

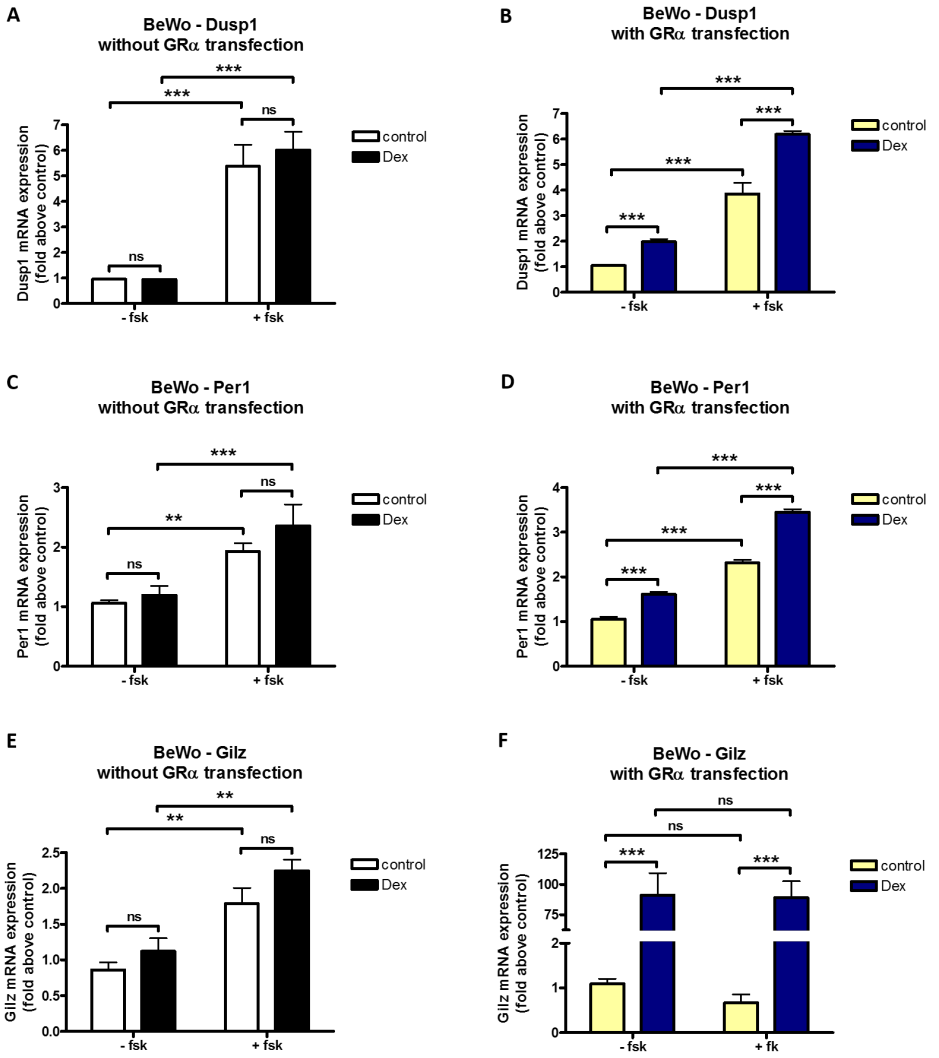

Figure 6.6: **Effect of dexamethasone and forskolin on GC-responsive gene expression in BeWo cells.** In the absence **(left graphs)** or presence **(right graphs)** of exogenous GRα, BeWo cells were treated with 10^{-6}M dexamethasone and/or 100 μM forskolin for 24 h before extracting the mRNA and performing qRT-PCR for Dusp1 **(A, B)**, Per1 **(C, D)**, and Gilz **(E, F)**. **(A, B)** Normalisation to 18S rRNA, **(C-F)** normalisation to RPLP0 mRNA, n=4, data are expressed as mean values ± SEM, p<0.01 (=**), p<0.001 (=***), ns=non-significant, 2-way ANOVA with Bonferroni post-test.

6.3.2 Effects of Dexamethasone and Forskolin on GRE-dependent Luciferase Transcription

Since the previous result suggested that in BeWo cells specific glucocorticoid-responsive genes were under the transcriptional control of forskolin and cAMP pathways, this hypothesis was further tested by performing a reporter-gene-assay with the MMTV-luc vector in BeWo (with and without GRα transfection) and HeLa cells after dexamethasone and/or forskolin treatment.

In HeLa cells, dexamethasone significantly increased luciferase activity by 58-fold compared to basal condition, whereas forskolin exerted a modest effect (4.3-fold increase) (Figure 6.7 A). Co-treatment with Dex and forskolin led to a further 52% increase compared to Dex treatment alone. This result suggested that in HeLa cells dexamethasone was the main driver of GR activation and cAMP pathways could further augment this effect exerting a synergistic action.

In native BeWo cells, dexamethasone did not induce a signal in the reporter-gene assay (Figure 6.7 B). Forskolin, however, significantly increased luciferase activity by 56-fold compared to control cells. In BeWo-GRα cells, dexamethasone significantly increased luciferase activity compared to control (Figure 6.7 C) and augmented forskolin-induced GR transactivation by 207% compared to forskolin treatment alone. Forskolin treatment significantly increased luciferase activity by 38-fold compared to control cells. This result suggested that in BeWo cells the absence of exogenous functional GR allows forskolin to control GC-responsive targets. Restoration of the GR levels then promotes a synergistic interaction between the two pathways.

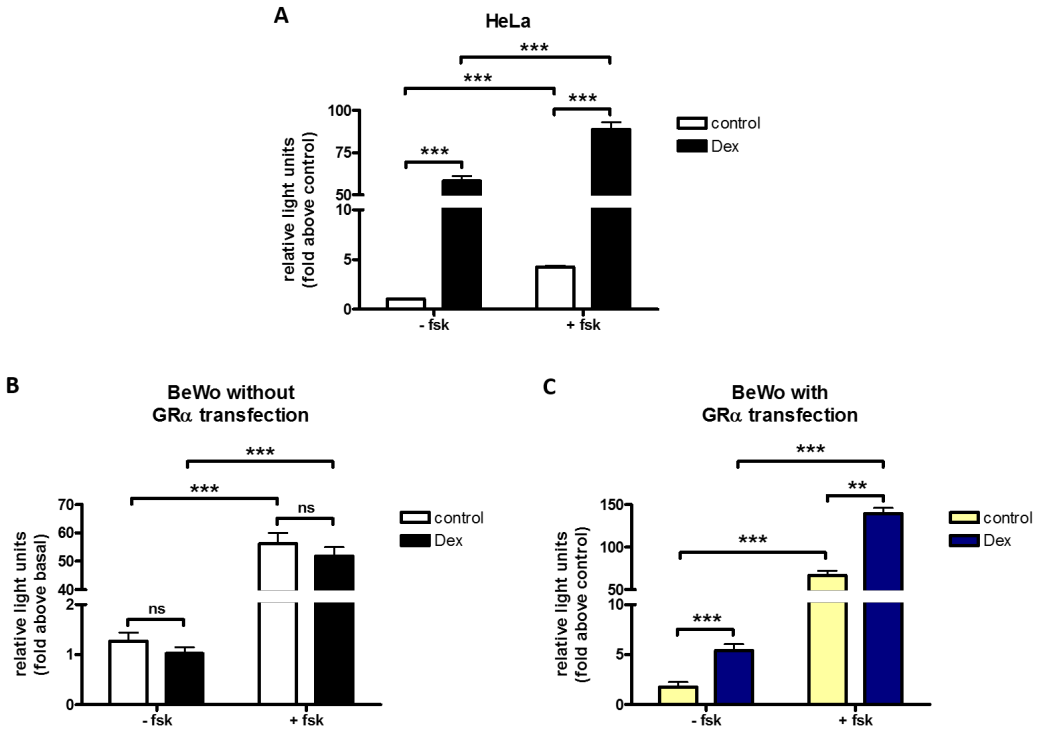

Figure 6.7: **Effect of dexamethasone and forskolin on GRE-dependent luciferase transcription.** HeLa cells were transfected with the MMTV-luc vector for 4 h **(A)** and treated with 10^{-6}M dexamethasone and/or 100 μM forskolin for 4 h before luciferase activity was determined. BeWo cells were transfected with the MMTV-vector with **(C)** and without GRα **(B)** for 4 h and treated with 10^{-6}M dexamethasone and/or 100 μM forskolin for 4 h before luciferase activity was determined. Samples were normalized to *renilla*-luciferase activity, n=3, data are expressed as mean values ± SEM, ns=non-significant, p<0.01 (=**), p<0.001 (=***), 2-way ANOVA with Bonferroni post-test.

6.4 Effects of Glucocorticoids in BeWo Cell Biology

Previous data showed that overexpression of GRα receptors in BeWo cells (= BeWo-GRα cells) restores cell responsiveness to glucocorticoids. Therefore, in my following experiments this modification was carried out.

6.4.1 Effects of Glucocorticoids on BeWo Cell Turnover and Differentiation

Glucocorticoids play an important role in differentiation processes of many different cell types [Ballard, 1979]. Thus, I investigated whether glucocorticoids can alter cell turnover including viability, apoptosis, and differentiation of BeWo-GRα cells.

6.4.1.1 Effects of Glucocorticoids on BeWo Cell Viability

BeWo-GRα cells were treated with dexamethasone for 24 and 48 h before assessing viability. Dexamethasone treatment did not lead to any changes in cell viability in BeWo-GRα cells (Figure 6.8).

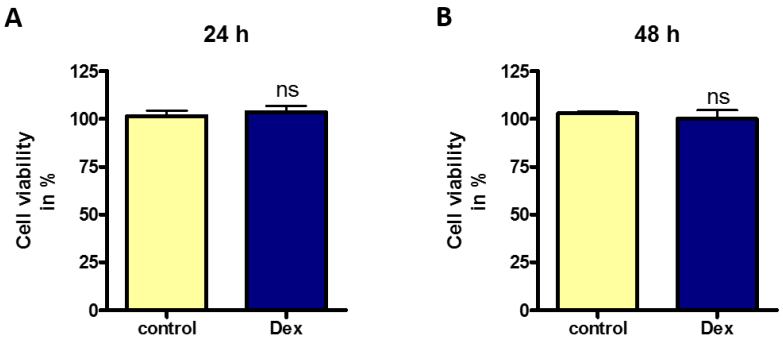

Figure 6.8: **Effect of glucocorticoids on BeWo-GRα cell viability.** BeWo cells were transfected with the GRα vector for 4 h and treated with 10^{-6} M dexamethasone for **(A)** 24 h or **(B)** 48 h before performing the MTT assay. n=3, data are expressed as mean values ± SEM, ns = non-significant, t-test.

6.4.1.2 Effects of Glucocorticoids on Apoptosis in BeWo Cells

BeWo-GRα cells were treated with dexamethasone for 24 and 48 h before assessing apoptosis. Figure 6.9 shows that dexamethasone did not influence the activation of caspase-3/7 in BeWo cells.

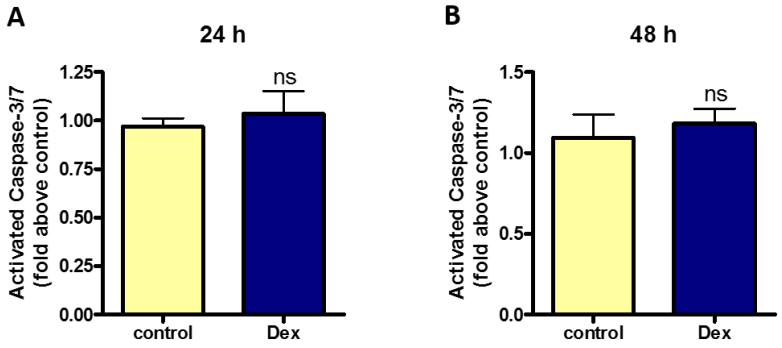

Figure 6.9: **Effect of glucocorticoids on BeWo-GRα cell apoptosis.** BeWo cells were transfected with the GRα vector for 4 h and treated with 10^{-6} M dexamethasone for **(A)** 24 h or **(B)** 48 h before measuring the activated caspase-3/7 with the ApoONE assay. **(A)** n=4, **(B)** n=3, data are expressed as mean values ± SEM, ns = non-significant, t-test.

6.4.1.3 Effects of Glucocorticoids on Differentiation of BeWo Cells

Effects of Glucocorticoids on Sync-1 and -2 Expression

BeWo-GRα cells were treated with dexamethasone and/or forskolin for 24 h before assessing Sync-1, -2, and their receptor expression. The previously observed (Figure 4.4 and 4.5) upregulating effect of forskolin on Sync-1, -2, and MFSD2 mRNA expression as well as the inhibitory effect of forskolin on ASCT2 mRNA was detected again (Figure 6.10 A-D). Dexamethasone treatment did not change Sync-1 mRNA expression (Figure 6.10 A). Dexamethasone minimally increased ASCT2 mRNA expression in differentiated cells (forskolin-treated for 24 h) (Figure 6.10 C). Interestingly, expression of Sync-2 and its receptor MFSD2 was slightly downregulated in undifferentiated BeWo cells by dexamethasone (Figure 6.10 B, D).

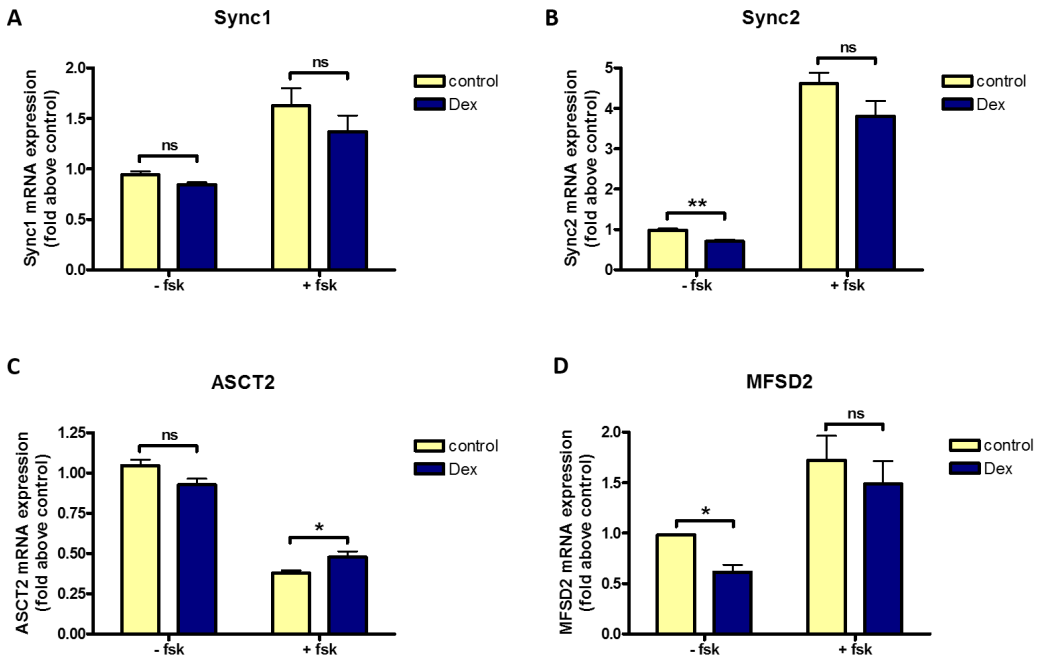

Figure 6.10: **Effect of dexamethasone and forskolin on Syncytins and their receptors in BeWo-GRα cells.** BeWo cells were transfected with the GRα vector for 4 h and treated with 10^{-6} M dexamethasone and/or 100 μM forskolin for 24 h before mRNA was extracted and qRT-PCR for **(A)** Syncytin-1, **(B)** Syncytin-2, **(C)** ASCT2, and **(D)** MFSD2 was performed. qRT-PCRs for Syncytin-1 and -2 were normalized to 18S rRNA, qRT-PCRs for ASCT2 and MFSD2 were normalized to RPLP0 mRNA, n=4, data are expressed as mean values ± SEM, $p<0.05$ (=*), $p<0.01$ (=**), ns=non-significant, 2-way ANOVA with Bonferroni post-test.

Effects of Glucocorticoids on hCG Secretion

BeWo-GRα cells were treated with dexamethasone (or cortisol) and/or forskolin for 24 h before measuring hCG secretion. As expected, the previously observed (Figure 4.8) forskolin-dependent induction in hCG secretion was confirmed (Figure 6.11). A small significant decrease of hCG secretion was observed in undifferentiated BeWo cells after Dex but not cortisol treatment, whereas no other effects in hCG secretion in differentiated BeWo cells could be detected.

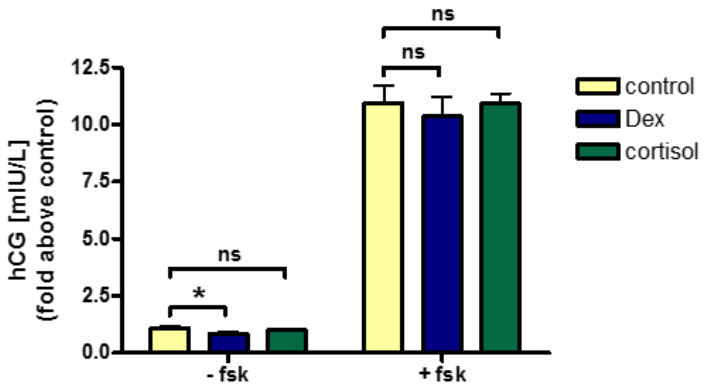

Figure 6.11: **Effect of glucocorticoids on hCG production in BeWo-GRα.** BeWo cells were transfected with the GRα vector for 4 h and treated with 10^{-6}M dexamethasone or 500 nM cortisol $\pm$ 100 μM forskolin for 24 h before measuring hCG in the supernatent. Samples were normalized to protein concentration, n=4, data are expressed as mean values $\pm$ SEM, $p<0.05$ (=*), ns=non-significant, 2-way ANOVA with Bonferroni post-test.

6.4.2 Effects of Glucocorticoids on Expression of Molecules involved in Stress Response and Glucocorticoid Action

BeWo-GRα cells offer a potentially useful model to investigate interactions between glucocorticoids and the placental CRH/CRH-R system.

The previously observed (Figure 4.11) forskolin-induced upregulation of CRH, CRH-R1 and CRH-R2 was again present in BeWo-GRα cells (Figure 6.12 A, B and C). The increased responsiveness of BeWo-GRα to Dex resulted in significant effects on CRH and CRH-R2 mRRNA expression: dexamethasone treatment led to a significant increase in CRH by 2-fold, and in CRH-R2 mRNA expression by 1.9-fold compared with control cells (Figure 6.12 A and C). The combination of dexamethasone and forskolin treatment did not lead to a further increase than the treatments carried out alone. CRH-R1 mRNA expression was not affected by dexamethasone treatment (Figure 6.12 B).

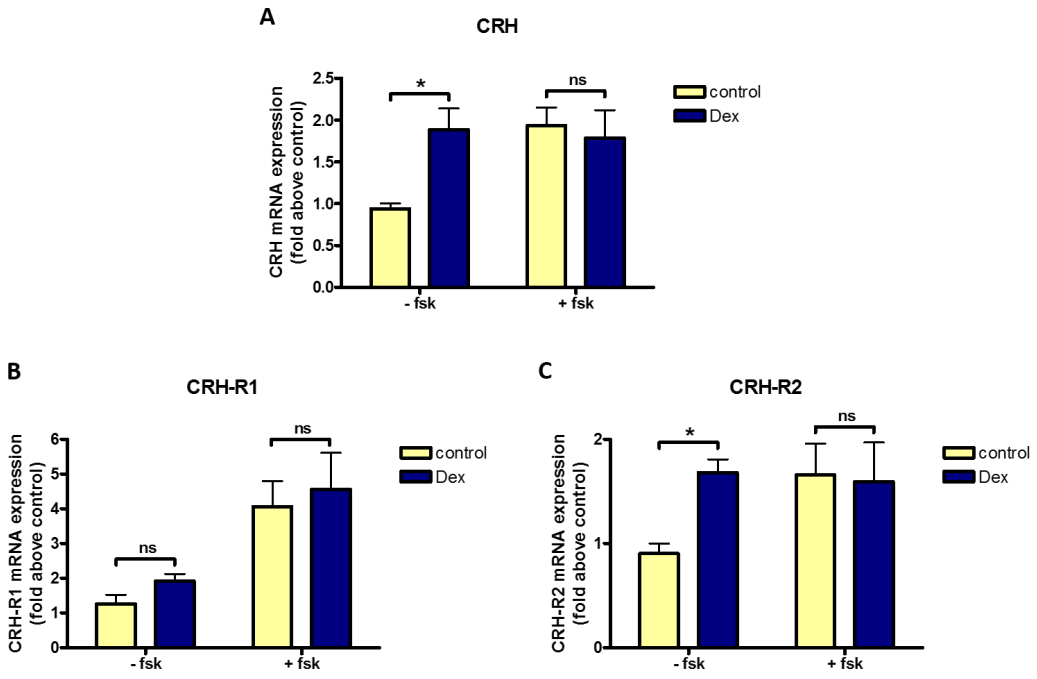

Figure 6.12: **Effect of dexamethasone and forskolin on CRH and its receptors in BeWo-GRα cells.** BeWo cells were transfected with the GRα-vector for 4 h and treated with 10^{-6} M dexamethasone and/or 100 μM forskolin for 24 h before mRNA was extracted and qRT-PCR for **(A)** CRH, **(B)** CRH-R1 and **(C)** CRH-R2 was performed. qRT-PCRs were normalized to RPLP0 mRNA expression, n=4, data are expressed as mean values ± SEM, $p<0.05$ (=*), ns=non-significant, 2-way ANOVA with Bonferroni post-test.

The previously observed (Figure 4.14, 4.17 and 4.13) forskolin-induced upregulation of 11β-HSD2 and BCRP mRNA expression as well as the forskolin-induced downregulation of MR mRNA was confirmed in BeWo-GRα cells (Figure 6.13 A, B and C). Dexamethasone treatment led to a small downregulation of 11β-HSD2 mRNA by 20% in undifferentiated BeWo-GRα cells, whereas there was no effect in differentiated cells (Figure 6.13 A). BCRP mRNA expression was not affected by dexamethasone treatment (Figure 6.13 B). MR mRNA expression was minimally, but significantly, upregulated in un- and differentiated BeWo-GRα cells (Figure 6.13 C). These results suggest that despite increased responsiveness to glucocorticoids these genes are not significantly regulated by glucocorticoids.

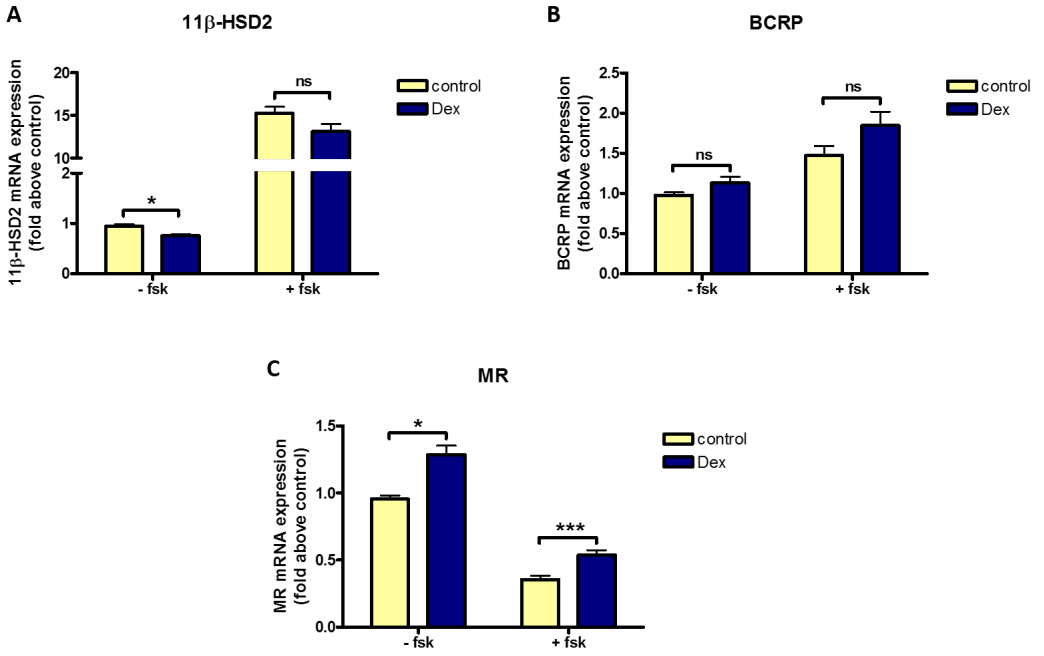

Figure 6.13: **Effect of dexamethasone and forskolin on 11β-HSD2, MR and BCRP in BeWo-GRα.** BeWo cells were transfected with the GRα-vector for 4 h and treated with 10^{-6} M dexamethasone and/or 100 μM forskolin for 24 h before mRNA was extracted and qRT-PCR for **(A)** 11β-HSD2, **(B)** BCRP and **(C)** MR was performed. qRT-PCRs were normalized to 18S rRNA expression, n=4, data are expressed as mean values $\pm$ SEM, $p<0.05$ (=*), $p<0.001$ (=***), ns=non-significant, 2-way ANOVA with Bonferroni post-test.

6.5 Discussion of Chapter 6

This chapter investigated the characteristics of BeWo cell responsiveness to glucocorticoids. My previous results (Chapter 5) raised the possibility that BeWo cells might be resistant to glucocorticoids. Furthermore, there was strong suggestion that the cAMP pathway appeared to be involved in the regulation of glucocorticoid-responsive genes. In order to investigate this, a reporter-gene-assay measuring binding of GRα to GRE-elements and transcription studies of several glucocorticoid-responsive genes were developed.

It has been shown that the placenta expresses the glucocorticoid receptor and that it is a glucocorticoid-responsive tissue, e.g. genes such as CRH, hCG, PAI-1, intergrins and GLUT1 are regulated by glucocortiocids in the placenta [Driver et al., 2001; Patel et al., 2003; Chan et al., 2003; Jones et al., 1989; Ringler et al., 1989a; Ma et al., 2002; Ryu et al., 1999; Hahn et al., 1999]. Previous experiments identified glucocorticoid sensitivity of term placental explants since dexamethasone treatment led to an increase of 11β-HSD2 and P-gp mRNA as well as FKBP5 protein expression (Chapter 3.4.1 and 3.4.3). However, it might be possible that cells other than trophoblasts are more responsive to glucocorticoids as Lee et al. [2005] showed only little expression of GR in syncytiotrophoblast and moderate expression of GR in cytotrophoblasts compared to the high GR expression in placental fibroblasts. Furthermore, they showed a higher phosphorylation status (at Ser203 and Ser211) of the endogenous GR when comparing placental fibroblasts with trophoblasts which might suggest presence of a more transcriptionally active GR in fibroblasts. Moreover, GR expression in placental cell lines such as BeWo and JEG-3 cells has been shown to be minimal [Chen et al., 1998; Stejskalova et al., 2013; Mark and Waddell, 2006] suggesting that immortalized trophoblast cell lines might be less responsive to glucocorticoids than primary placental tissue.

The GRα form of the glucocorticoid receptor is the predominant form in the term placenta [Johnson et al., 2008; Mparmpakas et al., 2014] and therefore this isoform was being analyzed in this study. Indeed, the expression of GRα in BeWo cells was only 10% of the GRα expression in HeLa cells which are highly responsive to glucocorticoids. Speeg and Harrison [1979] detected even lower amounts (2%) of GRα in BeWo cells compared with HeLa cells. Therefore, I studied the hypothesis that an increase in the GRα amount could restore BeWo cell responsiveness to glucocorticoids.

In HeLa cells, dexamethasone treatment led to a downregulation of GRα mRNA by 41% which is in agreement with the findings from Cidlowski and Cid-

lowski [1981], Burnstein et al. [1990], Burnstein et al. [1994] and Webster et al. [1997]. For example, Webster et al. [1997] detected a downregulation of GRα mRNA of 60% after 100 nM dexamthasone treatment in COS-1 cells. Some studies suggest that in placental explants, treatment with dexamethasone also leads to a decrease of GRα mRNA [Johnson et al., 2008]. However, in BeWo cells, endogenous GRα mRNA expression was not regulated by dexamethasone. This finding is in agreement with Mparmpakas et al. [2014] who showed that cortisol (10 - 1000 nM) does not change the GRα mRNA expression in BeWo cells. Studies in glucocorticoid resistant cells also showed that mRNA expression of GRα is not regulated by glucocorticoids [Sai et al., 2011; Tao et al., 2013] suggesting a correlation between glucocorticoid sensitivity and the regulation of GRα expression. Interestingly, Webster et al. [1997] observed that mouse GR which is mutated at several phosphorylation sites abolishes the dexamethasone-induced downregulation of the GR. Possibly, mutations at phosphorylation sites of the GRα might be a reason for the impaired glucocorticoid dependent suppression of endogenous GRα in BeWo cells and could be investigated in future studies.

The initial reporter-gene-assay experiments tested the capacity of the GRα receptor to bind to GRE elements encoded on the MMTV-luc vector. Treatment of HeLa cells with dexamethasone led to a strong binding of their endogenous GR to the MMTV-luc vector confirming their high responsiveness to glucocorticoids which has been shown by Nader et al. [2009]. This strong response could be enhanced with overexpression of exogenous GRα, but as the endogenous GR already produced a strong signal, this co-transfection was not necessary in the subsequent experiments. The endogenous GR of the HEK 293 cells also gave a response to dexamethasone treatment, however the intensity was only 2% of the HeLa cell signal confirming the relative low sensitivity of HEK 293 cells to glucocorticoids. Co-transfection of GRα into HEK 293 cells rendered them highly responsive to glucocorticoids, this strategy was also undertaken by several other groups [Kratschmar et al., 2012]. In contrast to these two cell lines, no increase in MMTV-luc activity could be detected after dexamethasone treatment in BeWo cells confirming the previous result that BeWo cells might be glucocorticoid resistant. Because overexpression of GRα in BeWo cells led to a strong signal after dexamethasone treatment (322-fold increase), it was concluded that transfection of exogenous GRα into BeWo cells restored their sensitivity to glucocorticoids. Similarly, transfection of GRα restores the glucocorticoid signalling in a SCLC (small cell lung carcinoma) cell line which is resistant to glucocorticoids and expresses an aberrant GR [Ray et al., 1994]. This approach (transfection of a GRα expression vector) has been previously used in studies in pla-

cental cell lines such a JEG-3 and BeWo cells [Chen et al., 1998; Stejskalova et al., 2013; Mark and Waddell, 2006].

Pathways controlling GC-responsive gene expression and roles of cAMP-driven pathways were investigated in BeWo and HeLa cells. In HeLa cells, results for Dusp1, Per1 and Gilz mRNA expression demonstrated their specificity of GC effects since regulation of these genes by the cAMP pathway seems to play an insignificant role compared to the strong regulation by dexamethasone. In contrast, the endogenous GR of the BeWo cells did not upregulate these genes after dexamethasone treatment which is consistent with their unresponsiveness in the reporter-gene-assay to dexamethasone. However, forskolin treatment led to a significant induction of those genes which suggests that the cAMP pathway controls the regulation of glucocorticoid-responsive genes in glucocorticoid resistant BeWo cells. Rendering the BeWo cells sensitive to glucocorticoids by transfection of exogenous GRα resulted in distinct and gene-specific effects. The gene Gilz was exclusively regulated by dexamethasone, confirming the complete restoration of glucocortiocoid sensitivity by transfection of exogenous GRα. In contrast, Dusp1 and Per1 glucocorticoid sensitivity seems to be only partially restored by transfection of GRα. This result suggests that the glucocorticoid and the cAMP signalling act independently but synergistically on the expression of some glucocorticoid-responsive genes. It might be possible that the GC regulation of these genes differs from each other, e.g. the promoter regions might contain different response elements such as simple GRE, composite GRE, competitive GRE or tethering mechanisms, which might explain the inconsistent effects of this experiment.

I investigated this phenomenon of signalling pathway cross-talk further using the reporter-gene-assay since the MMTV-luc reporter contains a well characterized composite GRE [Guido et al., 1996] (Figure 2.2). As expected, native BeWo cells did not respond to dexamethasone treatment, whereas HeLa exhibited a strong response signal. Similar to the gene transcription studies, forskolin only minimally transactivated GR in HeLa cells, whereas a strong effect of forskolin on GR transactivation was observable in BeWo cells in the absence as well as in the presence of exogenous GRα. The finding that the cAMP pathway is involved in the regulation of glucocorticoid-responsive genes has been identified by several groups. In many cellular systems, the cAMP/PKA pathway appears to enhance GR signalling. In particular, this synergistic action is well documented in neuronal models of disease. For example, Pace et al. [2011] showed that forskolin treatment augments the dexamethasone-induced MMTV-luc activity in HT22 mouse neuronal

cells, while inhibition of the PKA pathway with the antagonist H89 attenuates the dexamethasone-induced MMTV-luc activity. Also, Lim-Tio and Fuller [1998] detected an enhancing effect of 8-Br-cAMP on cortisol-induced MMTV-luc activity in renal LLC-PK1 cells. And Doucas et al. [2000] demonstrated a complex assembly of GR involving the catalytic subunit of PKA (=PKAc) in HEK 293 and HeLa cells as well as that an overexpression of PKAc in the monkey kidney CV-1 cell line enhances GR activation further in the presence of dexamethasone. They also observed a decrease in dexamethasone-induced MMTV-luc activity in the presence of a PKA inhibitor (H89) in CV-1 cells. In agreement with these findings, HeLa as well as BeWo cells (in the presence of exogenous GRα) showed a synergistic activity of dexamethasone and forskolin on MMTV-luc expression in my studies. Miller et al. [2002] investigated the effect of the PDE type 4 inhibitor rolipram which inhibits the breakdown of cAMP by phosphodiesterase (PDE) type 4 on GR function: they observed that rolipram treatment leads to a higher MMTV-luc activity without and with dexamethasone treatment in mouse fibroblast and rat glioma cells suggesting a possible action on GR in the absence of ligand. Also other groups observed a ligand independent transactivation of GR. For example, Eickelberg et al. [1999] observed an activation of GR after treatment with dibutyryl- and 8-bromo-cAMP in lung fibroblasts. Comparable to those studies is the result of the BeWo and HeLa cells which showed a forskolin-induced transactivation of the GRα in the absence of dexamethasone.

There are a number of potential interactions that might explain how the cAMP/PKA pathway is involved in the GR function. For example, PKA can phosphorylate the GR [Haske et al., 1994] leading to increased GR functionality, PKA leads to increased binding of GR to DNA [Rangarajan et al., 1992], or PKA can lead to a more stable mRNA transcript and a higher expression of GR [Dong et al., 1989; Peñuelas et al., 1998]. The latter explanation might not be relevant for BeWo and Hela cells as forskolin treatment did not lead to a change in GR mRNA expression in BeWo and HeLa cells unlike the observation by Dong et al. [1989]. This different result might be explained by the different cell lines used. However, it might be possible that phosphorylation processes of GR by PKA render the GR transcriptionally more active in BeWo and HeLa cells. Investigation of the phosphorylation status of the endogenous as well as transfected exogenous GRα after forskolin with or without dexamethasone treatment in BeWo cells could be a possible future research direction. Due to the lack of a CRE element in the MMTV-luc vector [Mulholland et al., 2003], CRE-induced transcription by cAMP is excluded as the cause for the observed stimulation of MMTV-luc transcription in BeWo cells. Mulholland

et al. [2003] also showed that the cAMP-induced MMTV-luc transcription is PKA-dependent, but CREB-independent. In order to test whether the forskolin-induced MMTV-luc transcription is independent of GRα, future experiments could use a GR antagonist (e.g. RU486) in BeWo cells with and without transfected exogenous GRα.

In conclusion, native BeWo cells seem to be resistant to glucocorticoids, but the overexpression of GRα (= BeWo-GRα cells) can restore the GC sensitivity. However, cAMP signalling appears to exert stronger effects on GRE-dependent transcription than glucocorticoids in BeWo-GRα cells. It is a common mechanism that cAMP signalling acts synergistically on GR activation, but in case of resistant cells to GC it seems that the relatively weak effect of the cAMP pathway (as seen in the HeLa cells) on the GC regulation of GC-responsive genes and GRE-dependent transcription events (possibly by GR activation) becomes stronger. A future study in primary placental tissue and primary trophoblast cells from different trimesters regarding cAMP signalling involvement in regulation of glucocorticoid-responsive genes would determine whether placental tissue is differently sensitive to GC depending on their developmental stage and whether this phenomenon is caused by a cAMP synergistic mechanism.

One reason for the glucocorticoid resistance in native BeWo cells besides the low expression of the GRα could be a mechanism upstream of the GR receptor that involves the enzyme 11β-HSD2 or transporter molecules such as P-glycoprotein and BCRP. All of these molecules are able to influence binding of active cortisol to its receptor, i.e. 11β-HSD2 by inactivating cortisol into cortisone and P-gp as well as BCRP by exporting active cortisol out of the cells. However, although 11β-HSD2 is highly expressed in the BeWo cells (Chapter 4.2.3), this option is unlikely since my previous results showed that downregulation of 11β-HSD2 did not alter the mRNA expression of glucocorticoid-responsive genes after cortisol treatment. Furthermore, dexamethasone is not being metabolized by 11β-HSD2 [Diederich et al., 2002]. Moreover, BeWo cells express only minimal amounts of P-gp, which makes it unlikely that this transporter can successfully export all exogenously added cortisol or dexamethasone molecules. As the BCRP is highly expressed in the BeWo cell line [Ceckova et al., 2006], efflux studies of cortisol and dexamethasone after manipulating BCRP expression might be of interest for future experiments.

The complex and extensive machinery of GR-associated proteins such as coactivators which are necessary for the active GR machinery such as p300/CBP as well as p/CAF and p160 family molecules (Figure 1.4) might exhibit defects in

expression in BeWo cells and this might explain GR insensitivity. For example, Grenier et al. [2004] showed in mouse Schwann cells that inhibition of the coactivators SRC-1 and SRC-2 by siRNA leads to an inhibition of dexamethasone-induced MMTV-luc expression by 60% and 40%, respectively. Waters et al. [2004] showed that overexpression of the repressor NCoR (nuclear co-repressor) leads to an attenuation of dexamethasone-induced GR transactivation in the A549 lung cancer cell line and they reveal that a GR resistant SCLS cell line expresses high levels of NCoR. In future experiments, expression levels of several coactivators and repressors could be characterized on mRNA and protein level in BeWo cells. Furthermore, chaperone molecules such as Hsp70, Hsp90 and immunophilins which are important for the maturation and translocation of the GR into the nucleus as well as for rendering the GR hormone-activatable [Japiassú et al., 2009] might have defects or might be low expressed in the BeWo cells.

Phosphorylation processes play an important role in the activation of the GR [Ismaili and Garabedian, 2004]. One or more of these phosphorylation sites could be mutated leading to an ineffective GR response of the BeWo cells. Mason and Housley [1993] showed that mutation of phosphorylation sites in the mouse GR changes the GR transcriptional activity. As mentioned above, in future experiments use of antibodies recognizing different phosphorylated GR forms could characterize the phosphorylation status of the endogenous GRα in BeWo cells (possibly in comparison with the phosphorylation status of transfected exogenous GRα).

Mutations at other sites of the GR might also explain the BeWo cell GC resistance. It might be possible that a mutation in the GR abrogates its ability to translocate to the nucleus. For example, Jewell et al. [1995] showed that transfected human GR which has a deletion of the hinge region containing the nuclear localisation signal exhibits a cytoplasmic distribution of the GR after dexamethasone binding in COS-1 cells. Immunocytochemical staining of the GR with and without dexamethasone treatment could test this possibility of an abolished translocation of the GR to the nucleus in the BeWo cells in future experiments.

Another possibility to explain the BeWo cell GC resistance is that BeWo cells might express high amounts of GRβ. GRβ has been shown to inhibit the transcriptional activity of GRα [Bamberger et al., 1995]. Transfection of GRβ into mouse hybridoma cells renders them insensitive to the action of glucocorticoids [Hauk et al., 2002]. Interestingly, it has been shown that the gender of the fetus has an influence on the resistance and sensitivity of the placenta to cortisol with male fetuses to be more resistant against cortisol programming effects. Saif et al. [2014] showed that there is an increased localisation of GRβ to the nucleus in male placentae which

might be the cause of their increased resistance to glucocorticoids. As the BeWo cells are male gender, this mechanism might play a role in the BeWo cell GC resistance. Indeed, Mparmpakas et al. [2014] showed that in term placentas the GRβ mRNA expression is only 2.5% of the GRα expression, whereas in BeWo cells the expression ratio from GRα to GRβ is approximitely 1:1. So, in future experiments, it is very important to perform quantitative PCR and westernblot experiments to compare the mRNA and protein levels of GRα and GRβ in BeWo cells (possibly in comparison to their levels in HeLa cells).

As the first reporter-gene-assay demonstrated the restoration of BeWo cell glucocorticoid sensitivity, transfection of exogenous GRα (= BeWo-GRα cells) was introduced in the experimental protocol to investigate effects of glucocortiocoids on BeWo cell turnover, differentiation and expression of molecules involved in the stress and glucocorticoid action molecular machinery.

This study did not find any effect of dexamethasone on BeWo-GRα cell turnover (specifically cell viability and activation of caspase-3/7). Regarding cell differentiation, a decrease of Syncytin-2 and its receptor was detected after dexamethasone treatment in undifferentiated cells, however in differentiated cells was no difference which suggests that the morphological fusion process initiated by forskolin treatment might probably not be affected by dexamethasone.

Several studies revealed a stimulating effect of glucocorticoids on hCG secretion of placental cells. In particular, Ringler et al. [1989a] showed that dexamethasone increases primary trophoblast hCG secretion and augments cAMP-induced hCG secretion after 48 and 72 h. Also, Höcker et al. [2004] showed that 48 h of prednisolone (a synthetic glucocorticoid) treatment leads to higher hCG secretion in primary trophoblast cells. And Ni et al. [2009] showed that 24 h of cortisol treatment increases mRNA levels of both α- and β-hCG as well as increases hCG protein secretion. In this study, no inducing glucocorticoid effect on hCG secretion was observed in the BeWo-GRα cells. This difference might be because of the fact that all of the before mentioned studies isolated primary trophoblasts from term placentae. It might be possible that glucocorticoid sensitivity of placental tissue increases during pregnancy as Speeg and Harrison [1979] detected a 27-fold increase of GR expression in term compared with first trimester placenta. Also, Hodyl et al. [2010] has shown that increased cortisol levels (observed in asthma patients) are associated with increased GR activity in placentae. As the cortisol concentration in fetal serum increases towards the end of pregnancy [Murphy, 1982], term placentae is potentially the condition of the highest glucocorticoid sensitivity and therefore,

effects of glucocorticoids in primary trophoblasts isolated from term placentae were observed in the above mentioned studies, but not in BeWo-GRα cells.

In the placenta, CRH is upregulated by glucocorticoids [Jones et al., 1989] and it appears that these two molecules are linked via a positive feedback loop mechanism. In BeWo-GRα cells, dexamethasone increased CRH mRNA expression which is similar to the observation of Wang et al. [2014b] who demonstrated that cortisol increases CRH mRNA levels in primary trophoblasts. Similar effects were observed for CRH-R2 mRNA expression in BeWo-GRα cells, in particular both dexamethasone and forskolin treatment led to an increase of CRH-R2. Interestingly, Wang et al. [2012] showed that in the placenta the CRH gene is constitutively regulated by an NFκB enhancer element which is driven by the RelB/NFκB2 transcription factor. They also showed that dexamethasone can stimulate the synthesis of RelB/NFκB2 and its nuclear translocation which might explain the positive regulation of CRH by glucocorticoids in the placenta. This finding suggests that the observed upregulation of CRH mRNA in BeWo-GRα cells by dexamethasone might be mediated by activation of NFκB signalling (which is in contrast to the first part of the studies presented in this chapter which concentrated on the GRE-dependent signalling pathways). Also, the fact that forskolin and dexamethasone did not exert synergistic effects on CRH mRNA expression in BeWo-GRα cells are in agreement with this explanation model because probably the CREB-induced and the NFκB-induced transcription machinery can not bind to the promoter region of the CRH gene at the same time. This result suggests that under specific experimental conditions the unique regulation of CRH by glucocorticoids in the placenta can be reproduced in the BeWo cells.

Regulation of components of the glucocorticoid action (11β-HSD2, BCRP, MR) in BeWo-GRα cells was only minimally influenced by dexamethasone. Several studies showed that glucocorticoids upregulate the expression and activity of 11β-HSD2 [Ni et al., 2009; Audette et al., 2010; Tzschoppe et al., 2011], whereas this effect was not detected in the BeWo-GRα cells. Again, these studies were carried out in primary term trophoblasts or in placental term explants which might represent a more sensitive system to the actions of GCs than BeWo-GRα cells.

In summary, HeLa cells showed a strong response to dexamethasone in the reporter-gene-assay as well as in the qRT-PCRs for glucocorticoid-responsive genes. In contrast to that, native BeWo cells were unresponsive to dexamethasone on reporter-gene and qRT-PCR level. However, after overexpression of GRα, BeWo cells produced a strong signal in the reporter-gene-assay after Dex treatment. A

modest upregulation of Dusp1, Per1, and a strong upregulation of Gilz was observed in BeWo-GRα cells after Dex treatment. Both results suggest that transfection of exogenous GRα can at least partially restore the BeWo cell capacity to respond to glucocorticoids. In BeWo-GRα cells, cell turnover and differentiation were not or only slightly affected by dexamethasone treatment, the expression of CRH and CRH-R2, but not CRH-R1, were upregulated by Dex, other components of the glucocorticoid action molecular machinery were not or only slightly affected.

An interplay of different signalling pathways in the regulation of the glucocorticoid response appears to be present in BeWo as well as in HeLa cells. In both types of cells, cAMP stimulation can activate glucocorticoid-responsive genes and can induce expression of a GRE-containing reporter-gene-vector, although this interaction is much weaker in HeLa cells.

Chapter 7

Summary and Conclusion

The understanding of placental biology and the placentation process are very important as alterations in these processes can lead to abnormal fetal development and affect long-term health. Pathological conditions associated with adaptation processes in response to physical as well as psychological stress responses can exert profound effects on placental biological mechanisms. These placental adaptation processes can impair placental efficacy and can cause placental insufficiency. Placental diseases such as pre-eclampsia and IUGR (Chapter 1.3.1) are examples of diseases associated with abnormal placentation processes. In addition, pre-existing maternal pathologies such as diabetes and obesity can also induce placental adaptation responses (Chapter 1.3.2). Thus, a detailed understanding of the placental dysfunction might help identify molecules or signalling pathways which could be targeted by future therapeutic investigations. The aim of this study was to explore the molecules involved in stress mechanisms, specifically the roles of CRH, glucocorticoids and 11β-HSD2 on some key basic cell biology processes of trophoblast cells.

During placentation and also during the course of pregnancy, fundamental cell biology processes such as proliferation, differentiation and apoptosis are activated in a coordinated manner to maintain trophoblast cell homeostasis (Chapter 1.2.5). In a finely tuned process, cytotrophoblast cells proliferate, differentiate and fuse with the adjacent syncytiotrophoblast cell layer. In the normal cell turnover of trophoblast cells, apoptosis occurs as shedding of syncytial knots from the syncytiotrophoblast. However, these processes seem to be altered in placental diseases as decreased differentiation and increased apoptosis are some of the hallmarks of placental pathologies [Langbein et al., 2008; Sharp et al., 2010; Heazell et al., 2011].

The syncytiotrophoblast fulfills several important functions such as nutrient transfer, hormone production and acts as a barrier to protect the fetus. The endocrine function of the placenta, especially secretion of human chorionic gonadotropin (hCG), progesterone and estradiol, is essential for a successful pregnancy (Chapter 1.2.7.2). The fetus and the placenta signal their demands via hormones to the mother and also maternal, fetal and placental hormones regulate placental and fetal development and growth (Figure 1.15). Endocrine activity might potentially be affected in placental pathologies. The syncytiotrophoblast also acts as a glucocorticoid barrier of which the enzyme 11β-HSD2 is the main component. 11β-HSD2 expression and activity which inactivates active cortisol into cortisone has been shown to be reduced in placental diseases such as pre-eclampsia and IUGR [Causevic and Mohaupt, 2007; Shams et al., 1998; McTernan et al., 2001; Dy et al., 2008; Börzsönyi et al., 2012]. As a result, excess glucocorticoids might reach the fetus which has detrimental effects on fetal development, but might also predispose the individuals to develop metabolic, cardiovascular and psychiatric disorders in later life (Chapter 1.3.3).

Important for the coordination of stress responses are the hormone CRH and its receptors CRH-R1 and -R2. As part of the HPA axis, CRH is the master regulator in controlling biological mechanisms to stress signals. In higher primates and humans, many regulatory roles for placental CRH have been proposed in placental biology (Chapter 1.1.2.2) and its levels rise towards the end of pregnancy. During all trimesters of pregnancy, glucocorticoids from the maternal circulation cross the placenta, but most of the molecules are inactivated by the enzyme 11β-HSD2. Near term cortisol produced by the fetus can reach the placenta and the activity of the placental 11β-HSD2 is decreased, both leading to higher cortisol levels towards the end of pregnancy which might be a the cause for the increasing CRH levels because of the positive-feedback mechanism of cortisol and CRH in the placenta. Alteration in the CRH expression as well as in the expression of the CRH receptors and the gluco- and mineralocarticoid receptor (GR and MR) might change the dynamics of the placental stress systems with possible changes in glucocorticoid action and potentially this might evoke placental adaptation or dysfunction.

In this thesis, the potential alterations of basic placental cell biology processes such as cell turnover (cell proliferation, viability, apoptosis), differentiation (expression of Syncytins and their receptors) and endocrine function (secretion of hCG, P4, E2) in placental explants and BeWo cells were investigated in several potential adverse conditions (in the presence of CRH, LPS and glucocorticoids). Moreover, the interplay of these processes with the enzyme 11β-HSD2 was explored

in BeWo cells. As the placental CRH system is possibly involved in regulatory placental adaptation processes under challenging conditions, the CRH responsiveness (expression of CRH and its receptors CRH-R1 and -2) was also investigated as well as the components which are involved in glucocorticoid action (GR, MR, 11β-HSD2, BCRP).

CRH levels have been shown to be increased in placental diseases [Laatikainen et al., 1991; Goland et al., 1993; Warren et al., 1992]. This feature might therefore be involved in the pathologies of the various placental diseases. Chapter 3 focused on investigating the effects of CRH on placental cell turnover and on endocrine function using the placental explant model. In addition, LPS was used to stimulate the TLR4 receptor of the placental cells which evokes an inflammatory response. Many placental diseases (pre-eclampsia, placental infection) as well as maternal pathologies (diabetes, obesity) are characterized by an exaggerated inflammatory response or a low-grade inflammatory milieu. Furthermore, also physiological processes such as the parturition process have been shown to involve activation of TLRs and induction of pro-inflammatory cytokines (Figure 1.17).

My studies showed that hCG hormone secretion of placental explants is influenced by the molecules CRH and LPS. In detail, in high hCG responders CRH has an inducing effect on hCG secretion by placental explants which is abrogated in the presence of LPS. This result suggests that CRH overexpression observed in placental diseases might be a mechanism of the placenta to augment reduced hormone secretion to a sufficient level. In contrast, in low hCG responders placental explant tissue seems to be hyperresponsive to stimuli as both CRH and LPS increased hCG secretion. No effect of CRH and LPS on placental explant cell turnover could be observed. Unfortunately, methodological complications of the placental explant led to the decision to focus subsequent research on the BeWo cell line.

The characterization of BeWo cells (Chapter 4) confirmed that the cAMP stimulator forskolin successfully induced cell fusion in this cell line to generate a syncytium-like state. Biochemical differentiation is characterized by upregulation of many molecules including hormones. Indeed, hCG and progesterone secretion were stimulated by forskolin which revealed the substantial endocrine function of syncytialized BeWo cells. Regarding the stress response molecular machinery, BeWo cell expression of CRH and its receptors was increased by forskolin suggesting an activation of placental stress regulatory function of fused BeWo cells. Furthermore, GR and MR were expressed in BeWo cells and MR expression was downregulated

by forskolin possibly protecting against glucocorticoid-induced MR effects. Components of the glucocorticoid barrier such as the enzyme 11β-HSD2 and transporter molecules which export cortisol were upregulated during BeWo cell differentiation suggesting a limitation of local glucocorticoid action.

The high expression of 11β-HSD2 in BeWo cells and their insensitivity to glucocorticoids offered the possibility to study additional roles of 11β-HSD2 that do not involve inactivation of cortisol to cortisone (Chapter 5). 11β-HSD2 appeared to have a pro-differentiating effect and might promote cell fusion in BeWo cells as 11β-HSD2 knockdown reduced mRNA levels of the Syncytin receptors which are involved in the fusion process. Moreover, 11β-HSD2 might also promote endocrine function as depletion of 11β-HSD2 decreased levels of secreted hCG and progesterone. During differentiation of trophoblast cells in the placenta, the apoptotic process is being activated, however possibly through a high expression of Bcl-2 the apoptotic cascade is arrested to allow formation of the syncytiotrophoblast (Chapter 1.2.6.3). As both hCG and progesterone have been shown to increase the anti-apoptotic Bcl-2 [Jasinska et al., 2006; Kajihara et al., 2011a,b; Liu et al., 2007], the study (in BeWo cells) might suggest a role of 11β-HSD2 in protecting against apoptosis through its role in the maintenance of sufficient hCG and progesterone production and might represent a possible mechanism of apoptosis prevention in the syncytiotrophoblast. Another role of 11β-HSD2 might involve the limitation of CRH production and sensitivity in BeWo cells as knockdown of 11β-HSD2 increased the expression of CRH and its receptor CRH-R2. Also over this pathway, apoptotic processes might be altered as CRH has been shown to limit progesterone secretion [Jeschke et al., 2005; Yang et al., 2006; Gao et al., 2012].

The regulation of glucocorticoid-dependent processes in the BeWo cells were explored as previous results demonstrated insensitivity of BeWo cells to cortisol, i.e. gluco- and mineralocorticoid-responsive genes seemed to be regulated by the cAMP pathway only (Figure 5.16 and 5.17 in chapter 5.5.2.2). Results of chapter 6 showed that native BeWo cells were unresponsive to glucocorticoids, but overexpression of exogenous GRα (= BeWo-GRα cells) restored their glucocorticoid sensitivity. As native BeWo cells were used in the studies of potential roles of 11β-HSD2 (Chapter 5), this strengthens the notion that the observed roles of 11β-HSD2 in cell turnover, differentiation and regulation of hormone secretion are independent of cortisol.

Possibly, increased local concentration of glucocorticoids might alter placental biology which could lead to placental adaptations as observed in placental

diseases. However, no influence of glucocorticoids on glucocorticoid-sensitive BeWo-GRα cell turnover, differentiation and endocrine function was detected. A potential increase in CRH sensitivity was observed as CRH and CRH-R2 mRNA expression was upregulated by dexamethasone in BeWo-GRα cells. The upregulation of CRH by dexamethasone might be due to a stimulated NFκB signalling.

Several potential causes of the glucocorticoid insensitivity of BeWo cells in addition to the relative low expression of GRα in native BeWo cells might be altered expression of coactivators, repressors and chaperones of the GR, potential alterations in the phosphorylation processes of the GR which activates its transcriptional action and a possible high expression of the GRα action inhibiting receptor GRβ. These options could be explored in future experiments.

In the absence of exogenous GRα, again regulation of glucocorticoid-dependent processes by the cAMP pathway was observed. In BeWo-GRα cells, a synergistic action of glucocorticoids and forskolin on glucocorticoid-dependent processes could be demonstrated, however the cAMP pathway appeared to be the stronger regulator. Whether this phenomenon might be a mechanism of trophoblast cells to become insensitive to glucocorticoids could be investigated in future studies.

Results of this thesis might suggest that CRH could play an important role as a potential regulatory hormone during placental adaptation to adverse stimuli as during BeWo cell differentiation, expression of CRH and cell responsiveness to CRH increases suggesting a higher sensitivity to CRH-induced regulatory processes and also the other conditions investigated (depletion of 11β-HSD2, increased sensitivity to GC action) revealed an increase of CRH and its receptor CRH-R2. Also a central role for the enzyme 11β-HSD2 in BeWo cells could be shown as results suggest that this enzyme is involved in many functions such as differentiation, hormone production and maintenance of cell viability. However, the effects of alterations in hormone production such as CRH, hCG and progesterone possibly evoked by modulation of 11β-HSD2 expression regarding basic cell biology processes such as cell differentiation and apoptosis seem to be diverse und further exploration is required.

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